UTERINE PAPILLARY SEROUS CARCINOMA

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Declaration of Originality

This thesis has been composed by me and has neither been presented nor accepted in any previous application for any other degree or professional qualification.

All work presented in this thesis was, unless acknowledged, carried out by myself.

Hannah Campbell

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My Parents: John and Maggie Monaghan, who together have shown me that by hard work and dedication, many dreams can be achieved.

Dedication

I dedicate this thesis to my husband Stephen, my sons Frederick and David and my daughter Zoe, whose impending birth concentrated my mind.

Abstract

Uterine Papillary Serous Carcinoma (UPSC) is an uncommon form of endometrial cancer comprising 10% of all endometrial carcinomas. It is a highly aggressive tumour and because of its poor prognosis, it accounts for a disproportionately large proportion of deaths from endometrial carcinoma. The reason for its aggressive behaviour is uncertain. The aim of the thesis was to investigate potential mechanisms of invasion of UPSC, using an immunohistochemical approach, using high-grade endometrioid endometrial carcinoma (EEC) as a comparator.

An audit of all cases of UPSC diagnosed in South East of Scotland over a 10 year period confirmed the poor prognosis of UPSC and showed that this was conferred even in those cases where the tumour was composed of 5% UPSC regardless of the additional tumour type. It also raised awareness of the need for accurate and complete surgical staging. Tissue microarrays (TMAs) were created from the central viable part of the tumour and the invasive edge of UPSC and EEC and were shown to be valid for studying expression of the markers involved in this study. There was intertumoural variation in expression of E- and P-cadherin, CD98, matrix metalloproteases (MMPs) -2 and -9 and ER and PR in both UPSC and EEC. ER status is known to affect expression of E cadherin, β catenin, CD98 and MMPs -2 and -9, and E cadherin levels were decreased and the other protein molecules all showed higher expression in EEC compared to UPSC. The results are consistent with the role that oestrogen plays in the development of EEC.

Intratumoural variation in expression of E cadherin, P cadherin, β catenin, CD98, Galectin-3, MMPs -2 and -9 was demonstrated, supporting the theory of a subclone of the tumour developing properties necessary for invasion. These data contribute to

the growing body of literature on UPSC, and address diagnostic and treatment uncertainties for the pathology, surgical and oncological teams.

Ethical Approval

The Medicine/Oncology II Research Ethics Committee of the Lothian research Ethics Committee gave ethical approval for this study.

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Abbreviations

List of the main abbreviations used in the text:

UPSC	Uterine Papillary Serous Carcinoma
EEC	Endometrioid Endometrial Carcinoma
TMA	Tissue MicroArray
MMP	Matrix Metalloprotease
ECIS	Endometrial Carcinoma in Situ
EmGD	Endometrial Glandular Dysplasia
ER	Estrogen Receptor
PR	Progesterone Receptor
EGFRF	Epidermal Growth Factor Receptor Family
EGFR	Epidermal Growth Factor Receptor
PDGFR	Platelet Derived Growth Factor Receptor
KIP	Kinase Inhibitor Protein
Cdks	Cyclin Dependent Kinases
E2F	Elongation Factor 2
Rb	Retinoblastoma Protein
TGF-β	Transforming Growth Factor-β
LOH	Loss of Heterozygosity
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
OSPC	Ovarian Serous Papillary Carcinoma
ECM	Extracellular Matrix
TIMP	Tissue Inhibitor of MMP

FIGO	International Federation of Gynaecology and Obstetrics
WART	Whole Abdominal Radiotherapy
IL-6	Interleukin-6
EDTA	Ethylenediamine Tetraacetic Acid
IHC	Immunohistochemistry
FISH	Fluorescent *In Situ* Hybridisation
PFS	Progression Free Survival
OS	Overall Survival
CCEC	Clear cell Carcinoma
TAH	Total Abdominal hysterectomy
BSO	Bilateral Salpingooopherectomy
APC	Adenomatous Polyposis Coli
LAT	L type Amino Acid Transporter
GAL	Galectin

Chapter 1

Literature Review of Uterine Papillary Serous Carcinoma.

1. Introduction

This chapter is a literature review of Uterine Papillary Serous Carcinoma (UPSC). Over the past few years there has been growing awareness of the importance of the diagnosis and consequently there has been an increase in the number of studies on UPSC, ranging from audits on presentation of UPSC through molecular studies, methods of diagnosis and numerous different potential treatment modalities.

1.1 Incidence and Age

Endometrial carcinoma is the most common pelvic genital malignancy in the western world {Creasman, 2003}. The majority of these cancers are of adenocarcinoma type, arising from endometrial epithelium. The most common subtype is endometrioid endometrial carcinoma (EEC), which accounts for about 80% of endometrial carcinomas {Burton, 1998;Santin, 2002;Santin, 2003}. UPSC is a highly aggressive variant of endometrial carcinoma and accounts for approximately 10% of cases {Burton, 1998}, but a much smaller proportion of Stage I endometrial carcinomas {Hendrickson, 1983;Bancher-Todesca, 1998;Dembo, 1985}. Patients present later than those with EEC, with a median age of diagnosis of 65 years for UPSC and 60 years for EEC.

1.2 Aetiology

Endometrial carcinomas are often split into 2 groups, determined by their clinical, histopathological and molecular features {Bokhman, 1983;Sherman, 1992}. These groups comprise: EEC (Type I) and non-endometrioid endometrial carcinomas (Type II). EEC usually arises from atypical hyperplasia of the endometrium of premenopausal, perimenopausal and postmenopausal women and is associated with nulliparity, obesity, exogenous oestrogen therapy and tamoxifen therapy. In contrast, UPSC usually occurs in elderly, postmenopausal women and is not associated with either atypical hyperplasia or oestrogen status. Indeed, most tumours arise from atrophic endometrium from a precursor lesion, Endometrial Carcinoma in Situ (ECIS), also known as Endometrial Intraepithelial Carcinoma, or Uterine Surface Carcinoma {Moll, 1996;Zheng, 1998;Spiegel, 1995;Sherman, 1992}. Zheng et al {Zheng, 2004} recently postulated that UPSC and ECIS might develop from dysplastic endometrial glands rather than de novo from normal postmenopausal endometrium. In a small series of cases they found endometrial glandular dysplasia (EmGD) in 17/32 (53%) uteri with UPSC compared with 1/60 (1.7%) uteri removed for EEC. EmGD was identified in 12/16 (75%) ECIS uteri. Areas of both EmGD and ECIS were found in 15/32 (47%) of the UPSC uteri. Transitions from either EmGD to ECIS or ECIS to UPSC were present in 8/32 (25%) of the UPSC cases. No transitions from EmGD to UPSC were identified in any hysterectomy specimen. EmGD was frequently found in endometrial polyps. There was no statistically significant difference between EmGD in a polyp (48%) and EmGD in non-polypoid endometrium (52%). In the majority of cases, EmGD was multifocal and involved superficial endometrial glands. However, single glandular involvement and surface

epithelial involvement of the endometrium was also seen. Although this is a small series, it does seem likely that there is some type of "interval" stage from normal endometrium to ECIS. However, further study is required in this area to elucidate the molecular changes occurring at these different stages.

1.2.1 Association with Breast Carcinoma

UPSC rarely expresses oestrogen receptors (ER) and progesterone receptors (PR) {Carcangiu, 1990}. Whilst some studies have demonstrated association of development of UPSC with tamoxifen therapy {Magriples, 1993}, other studies were unable to reproduce these findings, observing a similar rate of type I and type II endometrial carcinoma in breast cancer patients regardless of tamoxifen use {Barakat, 1994}. Geisler et al {Geisler, 2001} found that patients with UPSC had an increased risk of either synchronous breast cancer or subsequently developing breast cancer. Since UPSC is not a hormonally driven tumour, this raises the question as to why these patients are at risk of developing breast cancer compared to other patients with endometrial cancer. In the study by Geisler et al {Geisler, 2001} there was no significant difference in the mean age of patients with UPSC versus EEC, therefore age alone could not account for the difference. A case report and a study of 12 women raised a possible link between carrying the BRCA1-2 mutations and the development of UPSC {Lavie, 2000;Hornreich, 1999}. Existing studies show conflicting results as to whether endometrial cancer in general and UPSC in particular have an increased incidence of BRCA 1-2 mutations {Lavie, 2004;Goshen, 2000;Biron-Shental T, 2006}. Although all of these studies have been small, with limited power to determine significance, the disparity in results suggests that the

observed association between UPSC and breast cancer may be due to the presence of mutations in other cancer predisposing genes, such as P53 {Goshen, 2000}.

1.2.2 Association with Lymphoma

A case report by Chang et al {Chang, 2004} described a patient undergoing chemoradiotherapy for Stage IV non-Hodgkin's lymphoma who developed UPSC. They postulated that the depressed host immunity might have had a role in the development of UPSC. However, it is most likely that depression of the immune system plays a small role in the development of UPSC, as otherwise chronically immunosuppressed patients including renal transplant patients and those with HIV/AIDS also should have an increased incidence of UPSC and this has not been noted in the literature. In contrast, a recent paper demonstrated decreasing expression of HLA-DR and increasing expression of CD4 in normal endometrium, ECIS and UPSC respectively {Tamiolakis, 2005}. The authors suggested that low HLA-DR caused an "immune attract" mechanism. However, they also concluded that this probably played a minor role in the development of UPSC.

1.3 Pathogenesis

The exact pathogenesis of UPSC is unknown. However, recently many molecules including oncogenes and tumour suppressor genes have been demonstrated in association with UPSC. This section gives a brief overview of these molecules and proteins.

1.3.1 Oncogenes

Oncogenes are derived from proto-oncogenes: cellular genes that promote normal growth and differentiation. They may be found on the cell membrane (as growth factor receptors), or in the cytoplasm involved in signal transduction or in the nucleus, where they play a role in the cell cycle.

1.3.1.1 Growth Factor Receptors

C-erbB-2 (also known as HER-2/neu) is a member of the epidermal growth factor receptor family (EGFRF). EGFR proteins function as cell surface receptors and comprise three separate regions; the extracellular ligand binding site, a hydrophobic transmembrane region and a highly conserved intracellular signalling area, which contains tyrosine kinase {Prenzel, 2001}. The tyrosine kinase constituent can be phosphorylated to initiate complex intracellular signalling cascades, which in turn control cell cycle, survival, proliferation and gene transcription {Prenzel, 2001}. The C-erbB-2 gene can be amplified causing increased responsiveness of the signalling pathways and thus increased cell division and proliferation of damaged cells. Alternatively deletions may occur in the gene resulting in permanent activation of the receptor and increased cell division and proliferation {Prenzel, 2001;Moscatello, 1995}. Studies have shown overexpression of C-erbB-2 and amplification of the gene in USPC {Santin, 2002;Santin, 2003;Santin, 2005;Santin, 2005;Macwhinnie, 2004} .

1.3.1.2 Proteins Involved in Signal Transduction

Platelet derived growth factor receptor (PDGFR) and Abl overexpression has been identified immunohistochemically in primary and recurrent EEC and UPSC {Slomovitz, 2004}. Both of these proteins play a part in signal transduction from the cell membrane receptor, through the cytoplasm to the nucleus. When PDGFR is activated, it activates *ras*, found on the inner surface of the cell membrane, which in turn is transformed into its activated state and thus triggers the MAP kinase pathway, and proliferation. Thus, in UPSC, when PDGFR is overexpressed, the *ras* pathway is increasingly activated. Abl is a member of the non-receptor associated tyrosine kinase family and has tyrosine kinase activity, which is regulated by inhibitory domains. The main drawback of immunohistochemical studies is that they can only determine the presence or absence of a protein. However, the study examining the immunohistochemical expression of PDGFR and Abl also demonstrated expression of phosphorylated PDGFR and Abl, suggesting that these proteins are present in UPSC in the activated state {Slomovitz, 2004}.

1.3.1.3 Cell Cycle Regulators

The ultimate outcome of all growth promoting stimuli is the entry of quiescent cells into the cell cycle. Therefore genes associated with the cell cycle and in particular with the G1/S interface are frequently altered in cancer. P27 belongs to the kinase inhibitor protein (KIP) family and this protein inhibits the cyclin dependant kinases (cdks) associated with the cyclins E and D {Lloyd, 1999}. These particular cyclin kinase complexes regulate cell proliferation by stimulating the phosphorylation of the retinoblastoma protein (Rb), causing the release and activation of elongation

factor 2 (E2F), a transcriptional factor. E2F facilitates the traverse of the G1/S checkpoint of the cell cycle. Figure 1.1 shows a diagram of the cell cycle courtesy of Cotran et al {Cotran RS, 2006} Copyright © Elsevier 2005.

UPSC displays a high incidence of p27 alterations, suggesting that p27 abnormalities play an important role in the development of UPSC {Schmitz, 2000}. Cyclin D1 overexpression has also been noted in a small percentage of UPSC cases {Schmitz, 2000;Stamenkovic, 2000;Moreno-Bueno, 2004}. Cyclin E overexpression also occurs in UPSC, and may be associated with gene amplification {Cassia, 2003}. High levels of cyclin E, cdk2 and cdk4 correlate with weak or absent ER expression and p16 and p21 expression is associated with low PR expression {Milde-Langosch K, 2001}.

Recent studies have demonstrated that loss of *cables,* a cdk binding protein, is associated with a high incidence of endometrial hyperplasia and endometrial adenocarcinoma. This has been demonstrated not only in low grade EEC, but also in high grade EEC, UPSC and clear cell carcinoma {DeBernardo, 2005;Zukerberg, 2004}. *Cables* plays a role in proliferation and differentiation of the cell and its activity appears to be increased by progesterone and decreased by oestrogen {Zukerberg, 2004}.

1.3.2 Tumour Suppressor Genes

In normal cells tumour suppressor genes inhibit cell proliferation. Therefore loss of tumour suppressor genes causes an increase in cell proliferation. Tumour suppressor genes may be found on the cell surface, under the plasma membrane, in the cytoskeleton, cytoplasm and in the nucleus.

The Cell Cycle

External signals (growth factors, integrins)

MYC, *RAS*, and other genes

DNA damage, cell stress

Cyclin D

+ CDK4

Feedback regulation

p53

Cyclin D/CDK4 (active complex)

MDM2

p16INK4

p21

RB phosphorylation in E2F/DP1/RB complex

p14ARF

Active E2F

Cyclin E transcription (also Cyclin A, DNA polymerases, and many other genes)

Cyclin E

+ CDK2

Cyclin E/CDK2 (active complex)

p27

S

G_1

G_2

M

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1.3.2.1 Cell Surface

1.3.2.1.1 Transforming Growth Factor β

Transforming growth factor β (TGF-β) is a member of the large family of structurally related cytokines that play an important role in controlling cell proliferation, differentiation, migration and apoptosis {Derynck, 2001;Siegel, 2003}. Somatic mutations in the TGF-β signalling pathway are associated with loss of proliferative control, malignant progression, invasion and metastasis {Vogelmann, 2005}. TGF-β binds to specific transmembrane serine/threonine kinase TGF-β receptors such as type II (TGF-βRII), which in turn transphosphorylates type I receptor (TGF-βRI). Receptors convey signals across the plasma membrane through intracellular effectors such as SMAD proteins, which are translocated to the nucleus where they act as transcription factors {Massague, 2000;Itoh, 2000;Miyazono, 2000}. SMAD proteins have been divided into 3 functional classes: receptor regulated (R-SMADS), common mediator (Co-SMAD), and inhibitory (I-SMADs) {Miyazono, 2000}. Representatives of the first class are directly phosphorylated by TGF-βRI and are involved in the various signalling pathways. SMAD2 and SMAD3 are effectors of TGF-β/activin signalling while SMAD1, SMAD5 and SMAD8 are involved in bone morphogenetic protein (BMP) signalling. Co-SMAD is critical for TGF-β/activin and BMP signalling cascades. Representatives of the third class of SMADs (SMAD6 and SMAD7) have the ability to inhibit the TGF-β signalling pathway {Shi, 2001;Massague, 2000}. TGF-β can play multiple roles in human tumorigenesis, behaving as a tumour suppressor at early stages and a tumour promoter at late stages of carcinogenesis {Reiss, 1999;Wong, 2001;Wakefield,

2002}. Studies showed TGF-βRII protein level was higher and SMAD 2 and SMAD 4 mRNAs were lower in infiltrating endometrial carcinomas compared to Stage Ia EECs {Piestrzeniewicz-Ulanska, 2004}. Liu et al {Liu FS, 2003} showed that although LOH of chromosome 18q21 (the location of the SMAD4 gene) was frequent in endometrial carcinomas, immunohistochemistry showed that inactivation of the gene occurred infrequently. Both of these studies examined a mixed group of endometrial tumours and neither attempted to separate expression of type I and type II endometrial carcinomas. Therefore the role of TGFβ specifically in UPSC is still uncertain.

1.3.2.1.2 E Cadherin

E cadherin is the most common cadherin in epithelial cells. It is made from 4.5 kb mRNA as a 135kDa precursor polypeptide, which is processed rapidly by proteolytic cleavage to the mature 120kDa form {Shore, 1991}. The E cadherin gene (CDH1) is found on chromosome 16q22.1 and it is thought to be a tumour suppressor gene, whose loss has been shown to cause tumour invasion and metastasis in various cancer models {Christofori, 1999}. In EEC loss of heterozygosity at 16q22 has been related to poor prognosis and CDH1 mutations and promoter hypermethylation have been found in a small percentage of cases {Kihana, 1996;Risinger, 1994;Moreno-Bueno, 2003;Saito, 2003}. In addition to abnormalities in promoters of the E cadherin gene, or the gene itself, the E cadherin-catenin complex can be disturbed by the action of TGF-β. Vogelmann et al {Vogelmann, 2005} demonstrated that TGF-β induced destabilisation of the E cadherin-catenin complex and thus dissociation of β catenin from α catenin and the actin cytoskeleton. They also demonstrated that the

mechanism involved phosphorylation of β catenin by PTEN. Signalling through the cadherin-catenin complexes is involved in the regulation of EGFR distribution {Deugnier, 1999}. There is decreased expression of E cadherin in atypical hyperplasia compared to normal endometrium and even less in EEC {Shih, 2004;Moreno-Bueno, 2003}. Studies have also shown that loss of E cadherin expression increases with increasing grade of the endometrial carcinoma, such that the most poorly differentiated tumours have the least E cadherin expression {Moreno-Bueno, 2003;Shih, 2004;Dvalishvili, 2005}. Studies have shown conflicting results of E cadherin loss in type I, compared to type II endometrial carcinomas {Demopoulos, 1999;Moreno-Bueno, 2003;Shih, 2004}.

1.3.2.2 Nucleus

Numerous studies of P53 have been performed on endometrial carcinoma. P53 is involved in apoptosis, a complex energy dependent mechanism that eliminates unwanted cells selectively with minimal disturbance to surrounding cells {Kerr, 1972}. No p53 alteration is found in atypical hyperplasia, the precursor of EEC, whereas P53 alteration is common in both ECIS and UPSC {Zheng, 1998 ;Busmanis, 2005}. These data suggest that p53 inactivation may occur at an early stage in the pathogenesis of UPSC. P21 is a downstream effector of p53 mediated G1 arrest after DNA damage and is sometimes used as an indicator of p53 function. Kovalev et al {Kovalev, 1998} demonstrated that although 78% of UPSC cases showed strong nuclear staining for p53, only 53% of these tumours had detectable p53 mutations. In addition, 70% of those tumours with mutated p53 had concomitant lack of p21 expression consistent with transcriptionally inactive p53, whereas the other tumours

had p21 staining suggesting p53 independent p21 expression. Of the cases which did not show any p53 mutation, more than half still showed strong positive nuclear staining, but lacked concomitant p21 expression. These findings suggest that nuclear expression of p53 in UPSC can be associated with both mutation dependent and independent type of p53 inactivation. In addition, p53 expression is associated with loss of ER and PR expression {Moll, 1996}. A recent paper demonstrated expression of p63 in ECIS and UPSC. P63 is a p53-homologous gene which codes for multiple protein isotypes, including full length forms with p53 like actions and truncated forms with p53 antagonistic actions {Idrees MT, 2006}. The authors also demonstrated that p63 expression correlated with p53 and therefore postulated that p63 mutations also occurred early in the development of UPSC.

1.3.3 Genes that Regulate Apoptosis

In addition to p53, genes and gene-protein products such as Bcl-2, Bax and Bcl-x are involved in the control of apoptosis {Crescenzi, 2000;Lu, 1996}. Bcl-2 is a proto-oncogene, first identified from the t (14-18) translocation, which occurs in follicular lymphoma {Chen-Levy, 1989}. Bax is a 21-kDa protein that shares homology with Bcl-2 and forms homodimers {Oltvai, 1993}. It is thought that Bcl-2 suppresses apoptosis through heterodimerisation with Bax, but that Bax/Bax homodimerisation triggers programmed cell death {Yin, 1994}. Through an alternate splicing mechanism the Bcl-x gene encodes two proteins that have opposing functions {Boise, 1993}. Bcl-x_L is the long form and inhibits apoptosis. The short form, Bcl-x_S, triggers apoptosis. Studies have shown that expression of Bcl-2, Bax and Bcl-x_L varies between normal endometrium, atypical hyperplasia, EEC, ECIS and UPSC

{Bozdogan, 2002;Busmanis, 2005}. Bcl-2 expression in normal endometrium was significantly less than ECIS or UPSC, but no difference was found between ECIS and UPSC, suggesting that Bcl-2 loss occurs early in the pathogenesis of UPSC {Busmanis, 2005}.

1.3.4 Genes that Regulate DNA Repair

UPSC less commonly shows microsatellite instability (MSI) compared to EEC {King, 1995}. Hereditary Nonpolyposis Colorectal Carcinoma (HNPCC) is an inherited condition, characterised by germ line mutations in the mismatch repair genes MLH1 and MSH2. Abnormal methylation or mutations in these genes lead to MSI. Broaddus et al {Broaddus, 2006} showed that in those patients with HNPCC who developed EEC, hypermethylation of MLH1 had occurred leading to MSI. However, in those patients with HNPCC, who went on to develop non-EEC, Mutations of MSH2 were more likely to be found.

1.3.5 Karyotypic Changes.

Loss of heterozygosity (LOH) at chromosome 1p occurs in more than 63% UPSC, rarely in EEC and not in ovarian serous papillary carcinoma (OSPC). This suggests the presence of a tumour suppressor gene on chromosome 1p, important in UPSC tumorigenesis {Arlt, 1996;Herzog, 2001}.

1.4 Presenting Features

Most patients with UPSC present in a similar way to those with EEC. The most common presenting symptom is abnormal vaginal bleeding. Abdominal symptoms

such as bloating, pain or change in weight are occasionally seen and these symptoms are more common in patients presenting at a later stage {Podratz, 2003}.

1.5 Macroscopic appearances

UPSC has similar macroscopic features to all endometrial carcinomas. It usually forms exophytic lesions within the endometrial cavity, with varying degrees of myometrial invasion. Occasionally UPSC may arise in a polyp or form a polyp. Hui et al {Hui, 2005} studied 40 cases of UPSC, where macroscopically, the tumour was confined to the endometrium. They found that UPSC involved an endometrial polyp in 35 cases (88%) and in 18 cases (45%) extra-uterine tumour was present. In those cases, which were surgically staged as Stage Ia, either confined to a polyp or the endometrium, the overall survival after an average of 26 months follow up was 94%. They did not find any difference in outcome within the Stage Ia category where tumour was either confined to a polyp or was confined to the endometrium. There are no macroscopic features present, which can help differentiate between USPC and any other type of endometrial carcinoma.

1.6 Microscopic Appearances

UPSC was first recognised as a clinical variant in 1947 but it was not until 1982 that Hendrickson et al published a full clinical and morphological description of the lesion {Hendrickson, 1982}. Their criteria for diagnosis included complex papillary architecture with tufted stratification of the lining epithelium, marked nuclear polymorphism, high nuclear to cytoplasmic ratio, macronuclei and high mitotic rate. Solid areas could also be identified {Hendrickson, 1982}. Psammoma bodies can be

present in up to 33% of UPSC (See Figures 2.1 to 2.4 in chapter 2). UPSC is often found along with other subtypes of endometrial carcinoma including endometrioid and clear cell variants. Sherman et al {Sherman, 1992} suggested that the term UPSC should be used when it comprised at least 25% of the tumour. However, to date no study has been performed determining the exact amount of UPSC needed to be present to give the poor prognosis associated with "pure" UPSC. Some tumours are diffusely infiltrative, whereas others have a pushing border. The significance of this is uncertain.

1.6.1. Immunohistochemistry

Like all endometrial carcinomas, immunohistochemistry (IHC) shows UPSC to express CK7 and focal Ca125. UPSC is associated with overexpression of p53 and IHC for p53 is sometimes helpful in confirming the diagnosis. However, antibodies to p53 are not helpful in distinguishing between EEC and UPSC as both carcinomas express p53 to a variable degree, with increasing expression seen in any type of higher grade tumours {Macwhinnie, 2004;King, 1995}. GLUT1, a membrane bound facilitative glucose transporter, whose expression is associated with tumour hypoxia, has been demonstrated in ECIS and UPSC {Idrees MT, 2006}. Therefore it may have a role in distinguishing ECIS from normal or reactive endometrial epithelium. However, as it has also been demonstrated in atypical endometrial hyperplasia and EEC, it would not be useful in distinguishing UPSC from EEC. Expression of WT1, a tumour suppressor gene, can be used to distinguish OSPC from UPSC as OSPC uniformly expresses nuclear WT1 and UPSC shows either no expression or cytoplasmic expression of WT1 {Euscher, 2005}.

1.7 Spread

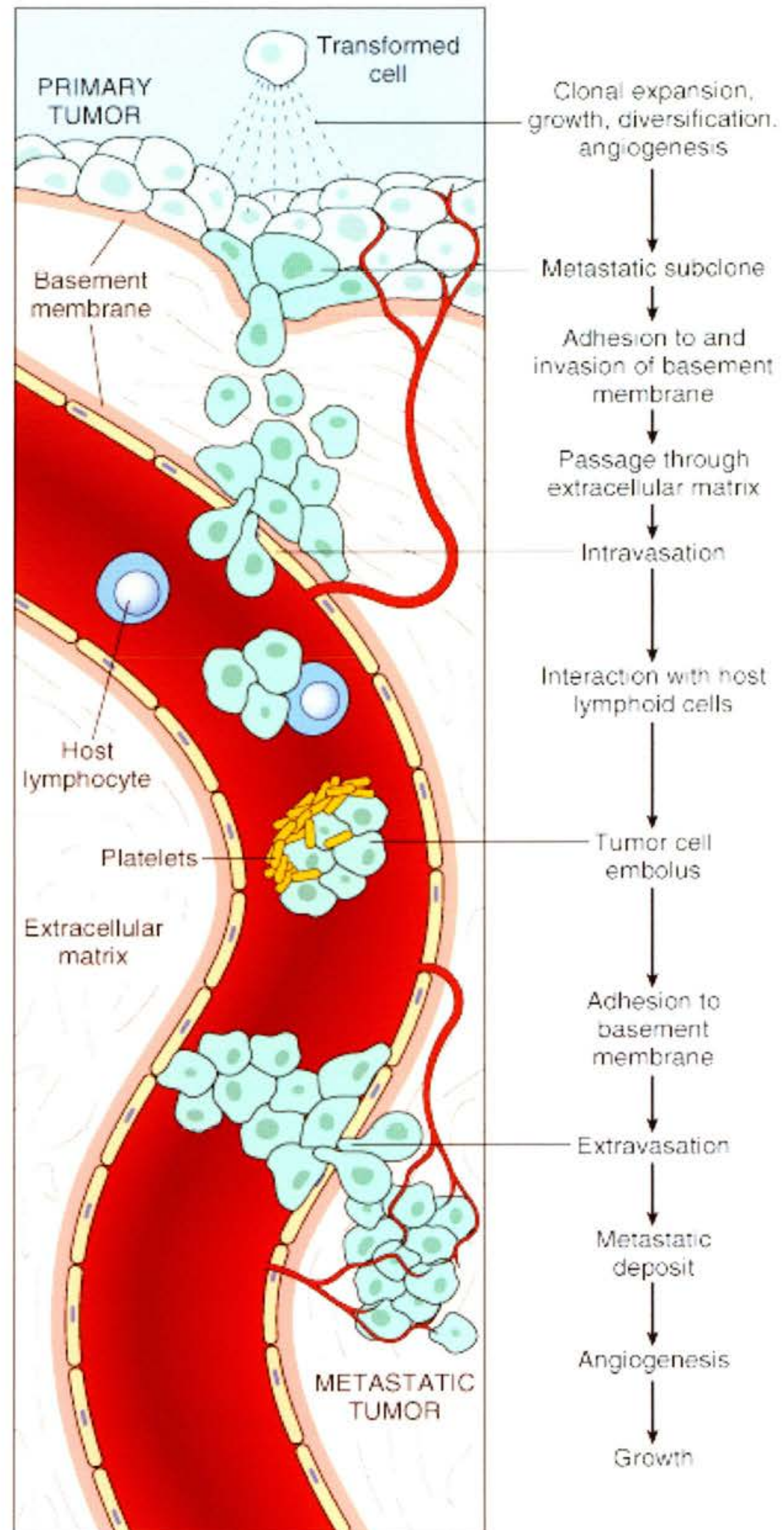
1.7.1 Pattern of Spread

UPSC resembles OSPC in its microscopic appearance and pattern of spread, but has a different molecular pathology {Hendrickson, 1982;King, 1995;Herzog, 2001 ;Zorn KK, 2005}. UPSC has a propensity for lymphovascular space involvement, stromal invasion and peritoneal spread {Lim, 2001}. It is upstaged at the time of surgery in 60% of cases {Gehrig, 2001;Jeffrey, 1986;Kato, 1995;Goff, 1994;Dunton, 1991}.

1.7.2 Mechanism of Spread

Invasion and metastasis involves many complex interactions and processes. Figure 1.2 shows the metastatic cascade, courtesy of Cotran et al {Cotran RS, 2006} Copyright © Elsevier 2005.

The Metastatic Cascade



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1.7.2.1 Detachment from Adjacent Cells

The role of E cadherin as a tumour suppressor gene has been previously discussed. This section focuses on its possible role in invasion of tumour cells. Cadherins are a widely distributed family of cell-cell adhesion molecules with varying degrees of tissue specific expression. E cadherin is found in all normal epithelia. As transmembrane cell adhesion molecules involved in cell-cell interactions, cadherin binding is homotypic and calcium dependent {Nose, 1988;Matsuzaki, 1990}. Intracellularly E cadherin is linked to members of the catenin family (β, γ, or α-catenin) through which it is connected to the actin cytoskeleton {Nathke, 1994;Hinck, 1994;Jou, 1995}. Cadherin-catenin complexes have roles in intercellular communication and modulation of cell function in both normal and malignant tissues. Failure to assemble the E cadherin-catenin complex or properly connect to the actin cytoskeleton results in loss of cell adhesion.

Decreased E cadherin expression levels have been observed in some "non EECs" {Moreno-Bueno, 2003}, and also has been associated with an increased risk of development of distant metastases {Pijnenborg, 2004}. However, nuclear localization of β catenin (associated with mutations in the β catenin gene) and mutations in the adenomatous polyposis coli (APC) gene were not predictive for recurrent disease. The exact role of E cadherin has not been fully elucidated and more work is needed in this area.

1.7.2.2 Attachment to Matrix Components

To penetrate the surrounding extracellular matrix (ECM), the tumour cells must first adhere to the ECM components. There is considerable evidence that receptor

mediated attachment of tumour cells to laminin and fibronectin is important for invasion and metastasis {Price JT, 1997;Ziober BL, 1996}. Normal epithelial cells express high affinity receptors for basement membrane (BM) laminin that are polarised to their basal surface. In contrast some carcinoma cells have many more receptors and they are distributed all round the cell membrane and there appears to be correlation between the density of receptors and invasion in colon and breast carcinoma {Ziober BL, 1996}. In addition to laminin receptors tumour cells also express integrins that can serve as receptors for many components of the ECM including fibronectin, laminin collagen and vitronectin {Vizirianakis, 2001;Yao, 1996}. Studies examining in vivo integrin expression in normal endometrium showed that β1 integrin expression was mostly seen in endometrial stromal cells {Castelbaum, 1997}. In addition, in vitro studies showed that progesterone treatment of oestradiol-primed cells resulted in increased expression of the α1β1 collagen-laminin receptor and suppression of the αvβ3 vitronectin receptor {Castelbaum, 1997}. Lessey et al {Lessey, 1995} found alteration of integrin expression between benign and malignant endometrial epithelium, with the α5β1 integrin, most commonly seen on benign endometrial stromal cells, being found in almost 20% of cases of endometrial carcinoma. They also showed that integrin expression correlated with steroid receptor status, as well as with grade, stage and depth of invasion {Lessey, 1995;Ziober BL, 1996}.

1.7.2.3 Invasion of Extracellular Matrix

Degradation of the ECM is an essential element of angiogenesis, cellular invasion and tumour metastasis. Matrix metalloproteinases (MMP), a family of zinc-

dependent endoproteinases, are widely accepted to play a role in these processes {Chang, 2001;Vihinen, 2002}. MMPs are normally expressed at a very low level in adult tissues, except in tissues that undergo remodelling such as cycling endometrium and wound healing. However, loss of control of MMP expression has been implicated in a number of diseases such as rheumatoid arthritis, osteoarthritis, chronic wounds and cancer {Chang, 2001;Vihinen, 2002;Stamenkovic, 2000;Parks, 1999;John, 2001;Jiang, 2002}. Based on their structure and substrate specificity MMPs are divided into several groups that include collagenases (MMP-1, MMP-3 and MMP-8), gelatinases (MMP-2 and MMP-9), stromeolysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP-26), metalloelastase (MMP-12) and membrane type matrix metalloproteinases (MT-MMPs) {Chang, 2001 ;Vihinen, 2002;Stamenkovic, 2000;Parks, 1999;John, 2001;Jiang, 2002 }.

The expression of MMPs is highly regulated at transcription, translation and secretion of latent enzyme, proenzyme activation and inactivation levels. The catalytic activity of MMPs is specifically controlled at least in part by their physiological inhibitors, the tissue inhibitors of MMPs (TIMPs), composed of TIMP-1, -2, -3 and -4 {Chang, 2001;Kugler, 1999;Jiang, 2002}. TIMP-1, -2 and -4 are secreted in soluble forms whereas TIMP-3 is insoluble due to binding to ECM proteins {Chang, 2001;Vihinen, 2002;Stamenkovic, 2000;Parks, 1999;John, 2001;Kugler, 1999;Jiang, 2002}. Although TIMP -4 appears to have restricted tissue expression and function, TIMP expression is found in many tissues and is regulated in coordination with MMPs. In addition to regulating MMPs, TIMPs have been demonstrated to have angiogenic and growth factor-like activity {Parks, 1999;John, 2001;Kugler, 1999;Jiang, 2002;Stetler-Stevenson, 1999}.

In the female reproductive tract tissues the expression of several MMPs and TIMPs is associated with menstruation, abnormal uterine bleeding, ovulation, embryo implantation, cervical ripening and parturition {Martelli, 1993;Rodgers, 1994;Matrisian, 1992}. Altered expression of MMPs (MMP-1, -2, -3, -7, -8, -9, -13) and TIMPs (TIMP-1, -2 and -3) have been shown to occur in endometrial adenocarcinoma and the results indicate increased expression of MMP-9 and MMP-7 is associated with myometrial invasion and lymph node metastasis respectively {Yabushita, 2000;Ueno, 1999;Moser, 1999}. In contrast MMP-2 expression is used to help differentiate high grade from low-grade endometrial carcinoma and MMP-9 in differentiating normal from sarcomatous endometrial stroma {Iurlaro, 1999;Liokumovich, 1999;Inoue, 1997}. Park et al {Park, 2001} demonstrated that cells from an endometrial adenocarcinoma cell line invaded by recruiting MMP-2 secreted by endometrial stromal cells and this was enhanced by the presence of beta oestradiol.

MMP-26 and TIMP-4 expression is elevated in endometrial carcinomas with the highest expression correlating with deeper myometrial invasion and grade 3 (high grade) tumours. There is no difference in expression between grade 3 EEC and USPC {Tunuguntla, 2003}.

1.8 Treatment

The most effective treatment for UPSC is surgery, and then depending on the stage of the disease, adjuvant chemotherapy, radiotherapy or both may be given. However, due to the relative rarity of the disease no chemotherapeutic regimes have been fully ratified in patients with UPSC.

1.8.1 Surgery

In 1988 the International Federation of Gynaecology and Obstetrics (FIGO) changed the staging criteria for endometrial carcinoma from a clinical to surgical system. To appropriately stage endometrial carcinoma by the FIGO system, the surgeon must perform a total extrafascial hysterectomy and bilateral salpingo-oophorectomy, obtain peritoneal washings, and sample any suspicious pelvic or para-aortic lymph nodes. Several authors have shown that 40% to 60% of clinical Stage I UPSC are upstaged at the time of surgical staging and lesions clinically confined to the endometrium are upstaged in approximately 60% of cases {Gehrig, 2001;Jeffrey, 1986;Kato, 1995;Goff, 1994}. Several studies have shown the presence of lymph node metastases in up to 50% of cases at the time of surgery {Huh, 2003;Slomovitz, 2003;Goff, 1994}. Therefore the use of prognostic factors appropriate for EEC including depth of myometrial invasion and lymph vascular space invasion may not apply to UPSC {Carcangiu, 1997;Carcangiu, 1992;Goff, 1994;Cirisano, 1999;Geisler, 1999;Gallion, 1989;Lee, 1991}.

The 1988 FIGO system does not include omental evaluation as part of the surgical staging criteria for endometrial carcinoma. However, this is frequently performed in cases where UPSC has been diagnosed preoperatively as UPSC and OSPC have a similar pattern of intra abdominal spread with a propensity to spread to the upper abdomen. Gehrig et al {Gehrig, 2003} performed a study on 52 women with UPSC who had had omentectomy as part of their surgical staging. Thirty-four of the omenta were macroscopically and microscopically benign, 2 contained microscopic metastases and 16 were macroscopically and microscopically involved with tumour.

The sensitivity of a visually negative omentum was 89%. The authors concluded that, with a microscopic metastasis rate of 4%, surgical sampling does not need to be included in the routine surgical staging of UPSC. However this would mean that 1 in 25 patients with UPSC would be under staged. Full surgical staging does appear to decrease local recurrences but using surgery as the sole therapy in UPSC is not useful due to the high recurrence rates, which mostly occur in the upper abdomen {Hendrickson, 1982;Jeffrey, 1986;Goff, 1994;Sutton, 1987}.

1.8.2 Radiation Therapy

The current rationale of treatment of endometrial carcinoma is to consider adjuvant pelvic radiotherapy in those patients with high-risk disease endometrial carcinoma (positive retroperitoneal lymph nodes, high histological grade or deep myometrial invasion). This is thought to reduce the risk of local recurrence, but has demonstrated less effect on overall survival as these patients usually die of distant disease {Gehrig, 2004;Elit, 2002;Bristow, 1999}. Huh et al {Huh, 2003} showed a similar overall survival in those patients with Stage I and II UPSC who were managed conservatively and those who received adjuvant radiotherapy suggesting that adjuvant radiotherapy may not have a role in those patients where the tumour is confined to the uterus. Whole abdominal pelvic irradiation (WART) has not been extensively studied, and consensus has not been reached regarding the benefits of this approach, but it is recommended, following resection due to the risk of upper abdominal dissemination {Lim, 2001}. However studies examining the survival of patients treated with post operative WART have shown conflicting results {Mallipeddi, 1993;Gibbons, 1991;Frank, 1991;Mehta, 2003}.

1.8.3 Hormonal Therapy

Progesterone therapy may be used empirically to treat metastatic EEC, as this is often a PR and ER positive tumour. However this has not been extensively used for patients with UPSC, as UPSC is usually an ER, and PR negative tumour {Umpierre, 1994;Carcangiu, 1990}.

1.8.4 Systemic Chemotherapy

Despite the microscopic similarity of UPSC to OSPC, UPSC does not show the same degree of chemosensitivity. However, the similarity of pattern of spread and recurrence to OSPC has led researchers evaluate similar treatments {Bristow, 1999}.

1.8.4.1 Platinum Based Chemotherapy

The most extensively evaluated treatment option in patients with UPSC is platinum-based chemotherapy, most commonly using cisplatin. Platinum-based agents act by cross-linking DNA, making it impossible for proliferating cells to replicate their chromosomes for mitosis. The cross-linked DNA activates DNA repair mechanisms, which ultimately induce apoptosis. Response rates associated with cisplatin-based chemotherapy range from 10% to 33% {Price, 1993;Chambers, 1996;Levenback, 1992}. However the combination of cisplatin, doxorubicin and cyclophosphamide chemotherapy is less effective in UPSC than in ovarian carcinoma {Price, 1993;Chambers, 1996}. Doxorubicin is a cytotoxic antibiotic and cyclophosphamide acts by alkylating DNA, thus damaging it and interfering with cell replication. To date, the results of intra-peritoneal cisplatin have failed to show dramatic results {Chambers, 1996}.

1.8.4.2 Taxol

Paclitaxel (Taxol), a member of the taxane group of drugs, is another treatment of ovarian cancer, which has also had some success in the treatment of UPSC. Single agent paclitaxel showed a tumour response in 77% of patients {Ramondetta, 2001}, and used in a neo-adjuvant fashion with cisplatin produced objective responses in 8 out of 9 patients {Zanotti, 1999}.

1.8.4.3 Topotecan

Topotecan is an established treatment in recurrent ovarian cancer, with response rates of 13% to 33% {McGuire, 2000;Swisher, 1997;Bookman, 1998}. Topotecan acts by blocking the action of topoisomerase I, an enzyme critical for cellular replication and repair. A pilot study assessing a 5-day regimen of topotecan as first line therapy in 15 women with UPSC found that 12 patients received topotecan as first-line therapy and 3 patients received it as second line treatment after failure of platinum therapy {Gore, 2002}. At median follow up of 13 months, 11 of the 12 patients who received first line topotecan (93%) remained in remission. Of the 3 patients receiving second line topotecan, 2 died and the other patient remained disease free at the end of the trial.

1.8.4.4 Herceptin

Several studies have showed amplification of the HER-2/neu (c-erbB2) oncogene in UPSC {Macwhinnie, 2004;Santin, 2003;Santin, 2002;Santin, 2005}. In addition, Santin et al {Santin, 2005} demonstrated that those UPSC patients with c-erbB2 amplification had a poorer prognosis than those without c-erbB2 amplification. On the basis of these findings as well as those of previous reports showing a positive in vivo correlation between the efficacy of Herceptin [R] therapy and the level of HER-2/neu overexpression by tumour cells, Herceptin [R] therapy may be a novel strategy in

a significant number of patients with chemotherapy resistant recurrent or metastatic UPSC {Santin, 2002}.

1.8.4.5 Imatinib Mesylate

Imatinib mesylate is a tyrosine kinase inhibitor that specifically targets c-kit, Abl and PDGFR. It has been shown to be effective in the treatment of patients with chronic myeloid leukaemia and gastrointestinal stromal tumours. These cancers are characterized by activating mutations of Abl and c-kit tyrosine kinases respectively. As both Abl and PDGFR are expressed in primary and recurrent UPSC, imatinib mesylate may be a useful treatment for these patients {Slomovitz, 2004}.

1.8.4.6 Summary

The benefit to patients from such multimodality therapy remains uncertain because no significant improvement after adjuvant therapy has been noted in the last decade {Bancher-Todesca, 1998;Tay, 1999}. However, there is clearly a need to continue investigating possible treatments due to the dismal prognosis of UPSC.

1.9 Prognosis

The outcome of endometrial carcinoma is influenced by the initial stage, and grade of the tumour. While early stage endometrial carcinoma is associated with relatively good cure rates, advanced stage disease is characterized by a poorer prognosis. Overall survival for women with surgically staged endometrial carcinoma ranges from 70% to 90% for those with Stages I and II disease and 5% to 60% for those with III and IV disease. However, when grouped by histological type, overall survival for women with EEC is 76% while survival for women with UPSC is much worse, ranging from 0 to 45% {Zanotti, 1999;Price, 1993;Gitsch, 1995;Creasman,

2003;Chambers, 1987;Carcangiu, 1992;Abeler, 1990}. Approximately 75% of women with EEC present with disease clinically confined to the uterus (Stage I), whereas 37-66% of women with UPSC tend to present with more advanced stage disease {Creasman, 2003;Gitsch, 1995}. Most series report survival rates of 35%-50% for Stages I and II and 0-15% for Stages III and IV UPSC {Jeffrey, 1986;Dunton, 1991;Gallion, 1989;Sutton, 1987;Abeler, 1990;Piura, 1998;Bristow, 2001}. Despite the best treatment attempts, relapse rates are reported to be as high as 50% to 80% {Christman, 1987;Chambers, 1987;Ramondetta, 2001;Hendrickson, 1982}.

Many studies have been performed in the attempt to identify factors that might be useful in predicting outcome of these patients. Population based data from the American National Cancer institute found that although the incidence of endometrial cancer was higher in white patients, black patients actually had a worse outcome {Hicks, 1998}. Santin et al {Santin, 2005}, found that earlier deaths were seen in those UPSC patients that overexpressed c-erbB2, black patients and those over 65 years of age at presentation. However, multivariate Cox regression showed that although short survival was significantly associated with c-erbB2 expression it was not associated with either race or age, suggesting that the c-erbB2 amplification caused much of the race disparity in survival in the patient population.

High serum levels of interleukin-6 (IL-6) are associated with, and expressed by, UPSC {Bellone, 2005;Santin, 2005}. IL-6 is a pleiotropic cytokine which is involved in the immune system, acute phase responses and has various effects on haematopoiesis {Hirano, 1998}. IL-6 regulates cell growth of a variety of human cancers and inhibits the antitumour effects of the immune system. High serum levels

of IL-6 correlate with shorter survival in patients with renal cell, prostatic and ovarian carcinoma {Blay, 1992;Nakashima, 2000;Scambia, 1995;Bachelot, 2003}.

The kallikrein family is large and its members are all proteases, which encode for trypsin-like or chymotrypsin-like serine proteases. Kallikrein -6 and -10 have been detected both in vivo and in vitro in UPSC {Santin, 2006;Santin, 2005}. Kallikrein 10 is implicated in the growth and invasion of breast, ovarian and prostate cancer {Diamandis, 2002;Goyal J, 1998}. Kallikrein-6, -10 and IL-6 may be useful in monitoring early disease recurrence and response to therapy.

Busmanis et al {Busmanis, 2005} did not find that p53 was a useful predictor for either survival time or disease stage. However, there is evidence that p73 expression profiles and p53 status are useful in differentiating between patients with good and poor prognosis. Despite P73 having significant homology to p53, it is not thought to be a tumour suppressor gene. The TP73 gene has 5 N-terminal isoforms including TAp73, DeltaNp73 and DeltaN'p73. Delta Np73 derives from an alternative promoter in intron 3 and lacks the transactivation domain of full length Tap73. It counteracts transactivation function, apoptosis and growth suppression mediated by wild-type p53 and TAp73 and confers drug resistance to wild type p53 harbouring tumour cells. Conversely down regulation of endogenous DeltaNp73 levels inhibits its suppressive action and enhances p53- and Tap73-mediated apoptosis {Zaika AI, 2002}. Using a support vector machine algorithm Becker et al {Becker K, 2006} demonstrated that TAp73, DeltaNp73 and DeltaN'p73 were significantly upregulated in all gynaecological tumours.

1.10 Summary

Uterine papillary serous carcinoma is a highly aggressive variant of endometrial carcinoma and accounts for approximately 10% of cases, but a much smaller proportion of Stage I endometrial carcinomas. It has a grim prognosis. This chapter has described what is currently known about UPSC and has focussed on the aetiology, pathogenesis and treatment of this aggressive cancer. Recently there have been several reviews of series of UPSC patients, predominantly focussing on the molecular pathology of UPSC or current treatment strategies, and all suggesting the value of a larger scale clinical trial {Slomovitz, 2003;Ramondetta, 2001;Zanotti, 1999;McGuire, 2000;Swisher, 1997}. To date there is no clinically proven treatment strategy for UPSC, and patients with this tumour receive ad hoc therapy, which has no proof of efficacy. A large scale clinical trial is important because all previous studies have been limited by the relative rarity of the disease; therefore the small numbers of patients seen in all centres and also the small numbers involved in these reviews have limited the power of the studies.

Chapter 2

Materials and Methods for Chapters 5, 6 and 7.

2.1 Tissues

2.1.1 Identification of Cases

Suitable cases were identified in three ways. Firstly, a search of the APEX computer system was made from 1992 to 2004. The codes used to identify suitable cases were T-84000 (endometrium), T-82920 (Total hysterectomy), T-82000 (Uterus), T-82900 (Uterus and cervix) and M-80103 (carcinoma), M-83803 (endometrioid carcinoma) and M-82603 (papillary carcinoma). The data that was recovered also included the patient's name, date of birth and the numbers accorded to the specimens by the Department of Pathology. Secondly a separate search was performed on the Department of Oncology Gynaecology database from 1994 to 2003, where the search parameter was any patients in whom a diagnosis of uterine papillary serous carcinoma (UPSC) had been made. This data was predominantly used for the audit into the diagnosis and management of UPSC. Lastly the paper records of the weekly regional Gynae-oncology multidisciplinary meeting were examined and those cases that on review were described as either UPSC or Grade 3 endometrioid endometrial carcinoma (EEC) were selected. The results from all three searches were combined. For the purposes of the audit on the diagnosis and management of UPSC, all of the cases from 1994 to 2003, selected by the oncology database were used. For the creation of the tissue microarrays (TMAs), all cases where blocks and remaining tissue were available were used. No patients or cases were excluded for any other reason.

2.1.2 Patient Demographics

2.1.2.1 Uterine Papillary Serous Carcinoma

The demographic details for those patients selected for the audit on the diagnosis and management of UPSC are described in Chapter 4. Seventy-eight patients were identified for the TMAs. Their demographics are described here. The median age of the patients was 68 years (range 49-89). Nine patients (12%) had a past medical history of breast cancer. No patients developed breast cancer after the time of diagnosis of UPSC. The FIGO staging system {Creasman, 1990} was used in describing the stage of disease. Table 2.1 shows the FIGO staging system. The numbers and percentages of each patient with each stage of disease are presented in Table 2.2. Hui et al {Hui, 2005} showed that UPSC confined to either an endometrial polyp or the endometrium had good prognosis. However, as only 4 of our UPSC cases were Stage Ia, these were not treated as a separate group.

2.1.2.2 Endometrioid Endometrial Carcinoma

Twenty patients with FIGO grade 3 EEC were identified. The median age of the patients was 66 years (range 46-85 years). No patients had either a past history of breast carcinoma, or developed it subsequently. The numbers and percentages of each patient with each stage of disease are presented in Table 2.2.

Table 2.1 FIGO Staging for Carcinoma of the Endometrium

Stage I	Tumour confined to uterine corpus
IA	Limited to endometrium
IB	Invasion less than half of myometrium
IC	Invasion more than half of myometrium
Stage II	Tumour involves cervix
IIA	Endocervical glandular (mucosal) involvement only
IIB	Invasion into cervical stroma
Stage III	
IIIA	Tumour invades serosal of corpus uteri and/or adnexae and/or positive cytological findings
IIIB	Vaginal metastases
IIIC	Metastases to pelvic and/or paraaortic lymph nodes
Stage IV	
IV A	Tumour invades bladder mucosa and/or bowel mucosa
IV B	Distant metastases, including intra-abdominal metastasis and/or inguinal lymph nodes

Table 2.2 Demographic Details of Patients Used to Create Tissue Microarrays.

	UPSC	EEC
Median Age (Range)	68 years (49-89) years	66 years (45-85) years
History of Breast Carcinoma (Patients)	9	0
Stage I	29 (37%)	14 (70%)
IA	4	0
IB	17	9
IC	8	5
Stage II	21 (27%)	1 (5%)
IIA	10	0
IIB	11	1
Stage III	17 (22%)	4 (20%)
IIIA	12	4
IIIB	2	0
IIIC	3	0
Stage IV	11 (14%)	1 (5%)
Total Patients	78	20

2.1.3 Tissue Samples

Archival tissue samples were selected from the Royal Infirmary of Edinburgh Department of Pathology, the slides were reviewed by HM and ARWW (a subspecialist gynaecological pathologist), the diagnosis of UPSC or EEC was confirmed and the appropriate paraffin blocks were selected. All tissue had been routinely fixed in 4% buffered formaldehyde and processed into paraffin blocks. The diagnostic features used to classify cases as UPSC and EEC are well described and are as follows {Robboy}:

2.1.3.1 Uterine Papillary Serous Carcinoma

At low power UPSC shows either papillary architecture or solid areas where the papillae are composed of broad fibrovascular cores with secondary or tertiary papillary processes and with prominent dropping off of cells into the lumen. The cells are generally rounded and the nuclei are pleomorphic with occasional multinucleation. In addition the nuclei tend to be centrally placed rather than lying adjacent to the basement membrane. Mitoses and foci of necrosis are common. Psammoma bodies are found in some cases (Figures 2.1 to 2.4).

Figure 2.1

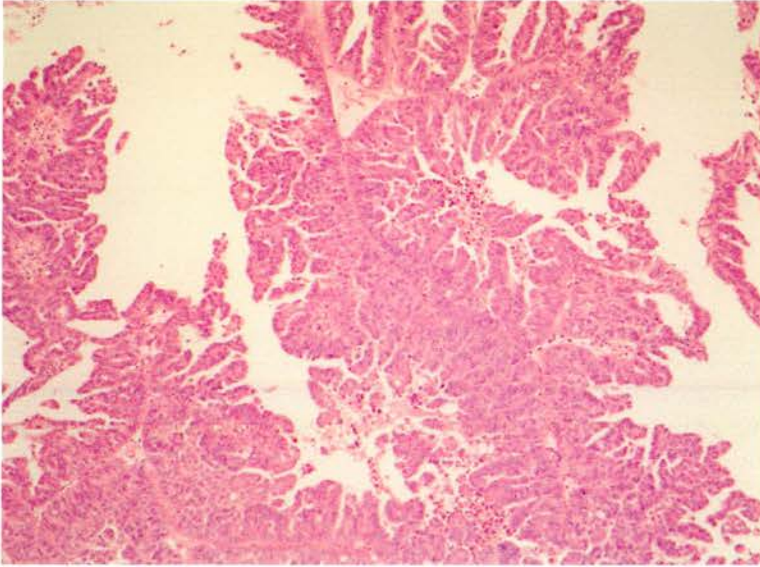


Figure 2.2

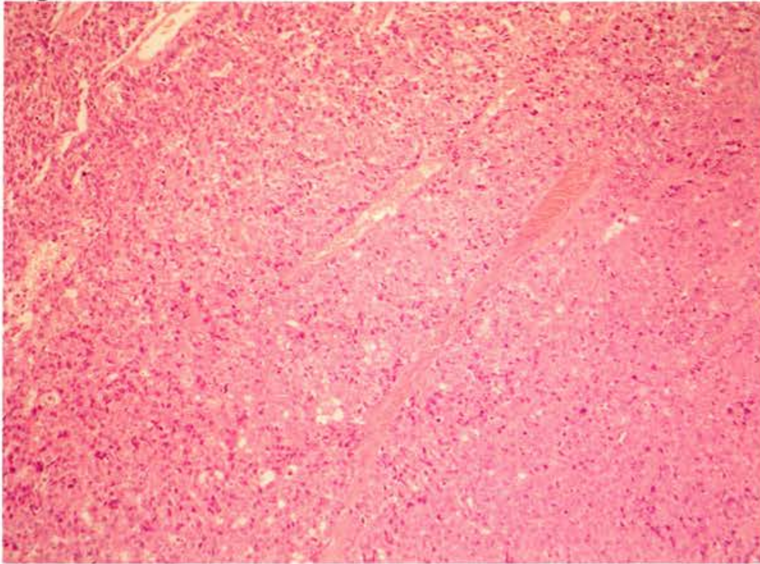


Figure 2.1. Papillary Architecture of UPSC (H&E x10).

Figure 2.2. Solid Architecture of UPSC (H&E x10).

Figure 2.3

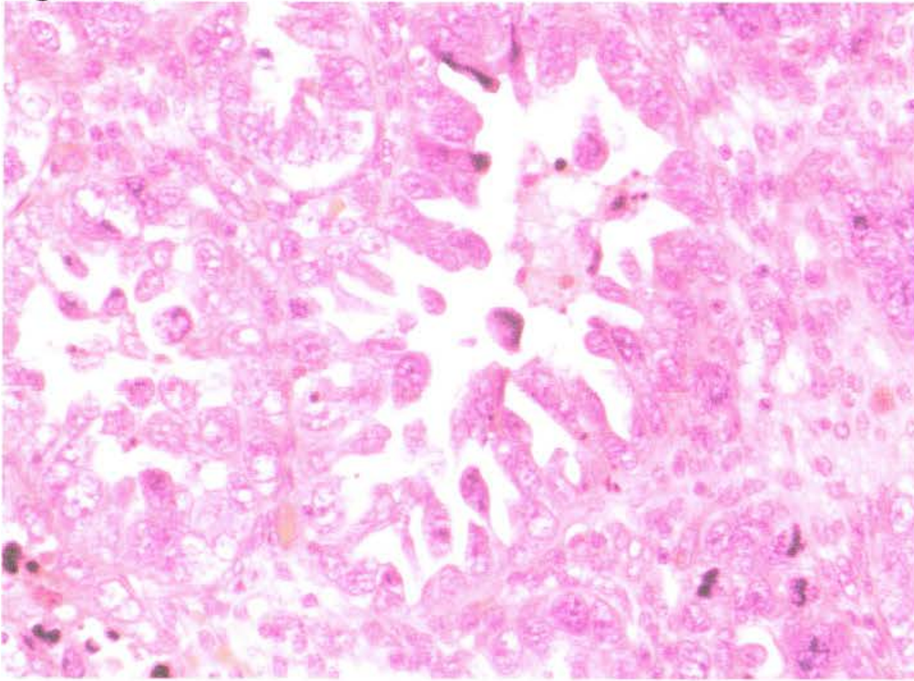


Figure 2.4

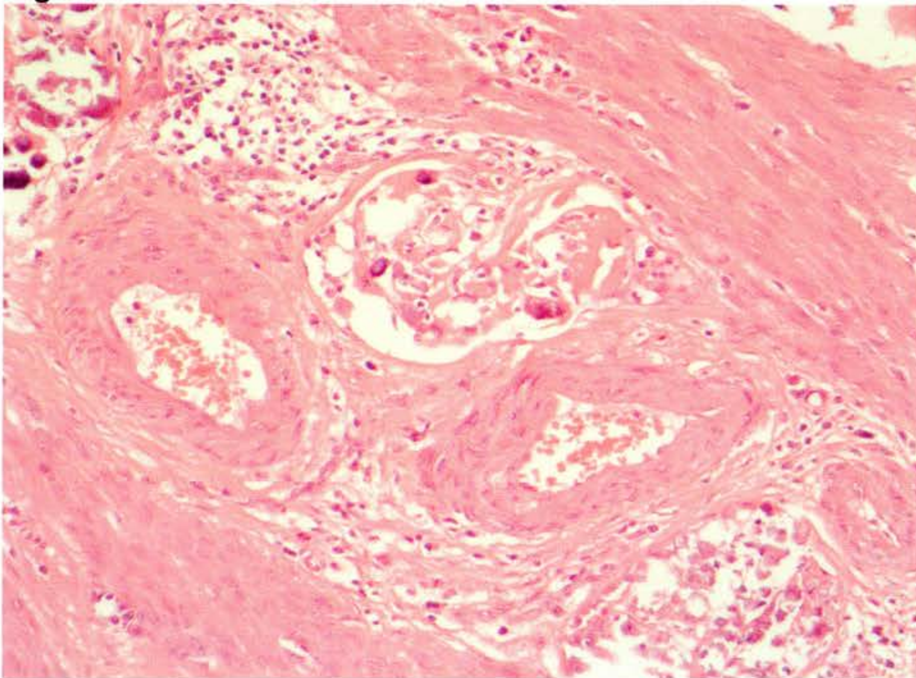


Figure 2.3. Pleomorphic Nuclei forming a papillary pattern in UPSC (H&Ex40).

Figure 2.4 Psammoma Bodies Lying within Tumour Adjacent to Myometrial Arteries (H&Ex20).

2.1.3.2 Endometrioid Endometrial Carcinoma

EEC can range from low to high grade. The well-differentiated (Grade 1) carcinomas can be difficult to distinguish from atypical complex hyperplasia. EEC typically has at least a focus of glandular differentiation lined by stratified columnar cells, with oval nuclei arranged at right angles to the basement membrane. As the carcinoma becomes more poorly differentiated (Grade 3), the prominent gland formation decreases and solid areas and sheets of cells are more apparent {Tavassoli, 2006} (Figures 2.5 to 2.8). EEC is typically divided into three grades, namely; Grade 1, Grade 2 and Grade 3, which are also referred to as well-, moderately- and poorly-differentiated respectively. The classification of grading developed by the World Health Organisation {Scully} is used and Table 2.3 shows the grading of EEC with architecture being the most important determinant. Significant nuclear atypia, described as round nuclei, variation in shape and size, variation in staining, hyperchromasia, coarsely clumped chromatin, prominent nucleoli, frequent mitoses and abnormal mitoses, will increase the grade by one, irrespective of the architectural features. Only cases of Grade 3 (high grade) EEC were selected for comparison with UPSC for this study.

At least one area from the viable central part and invasive edge of the tumour were then marked by a circle (Figure 2.9).

Figure 2.5

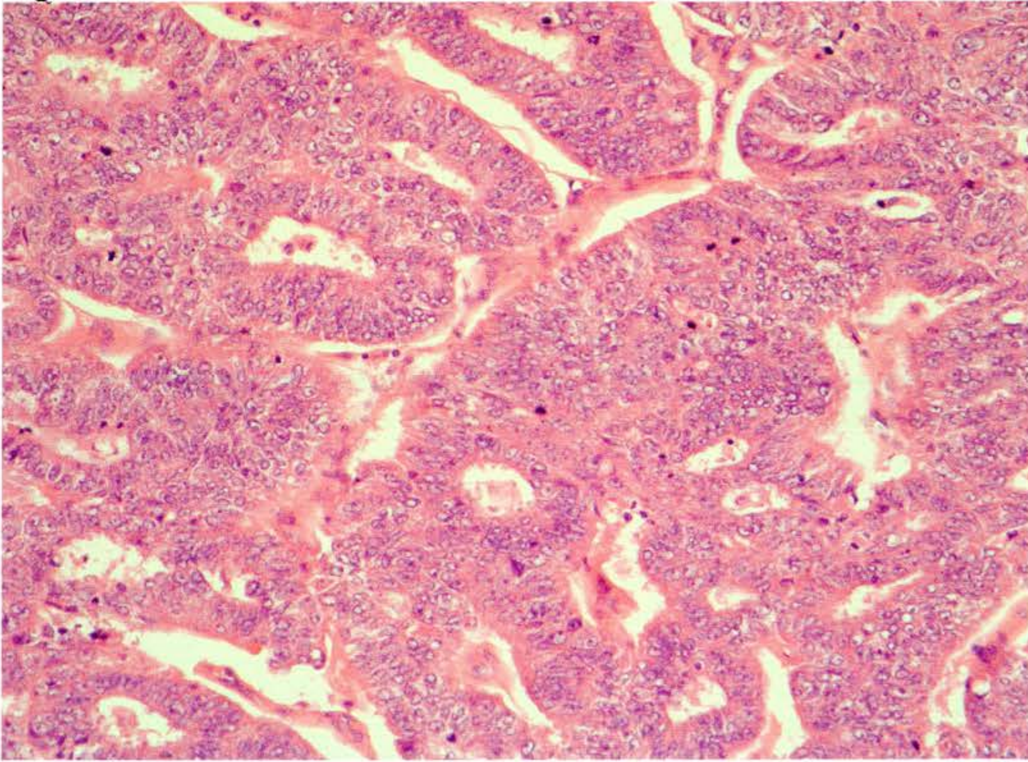


Figure 2.6

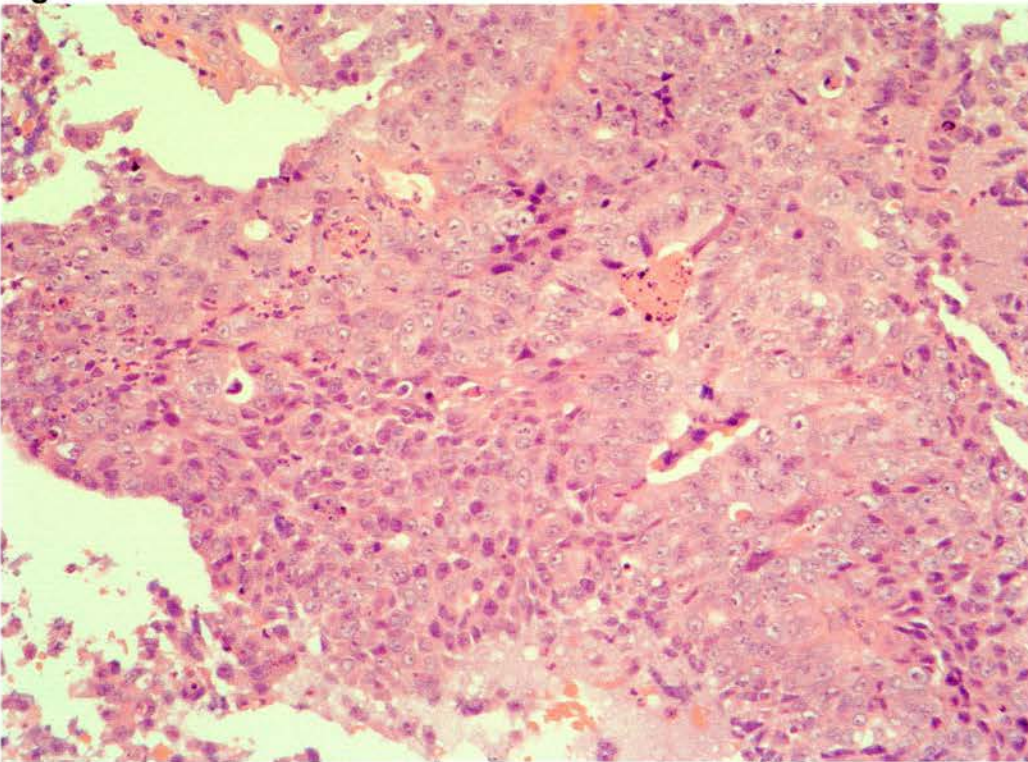


Figure 2.5. Classical Gland Formation of EEC (H&E x20).

Figure 2.6. Solid Architecture of EEC (H&E x10).

Figure 2.7

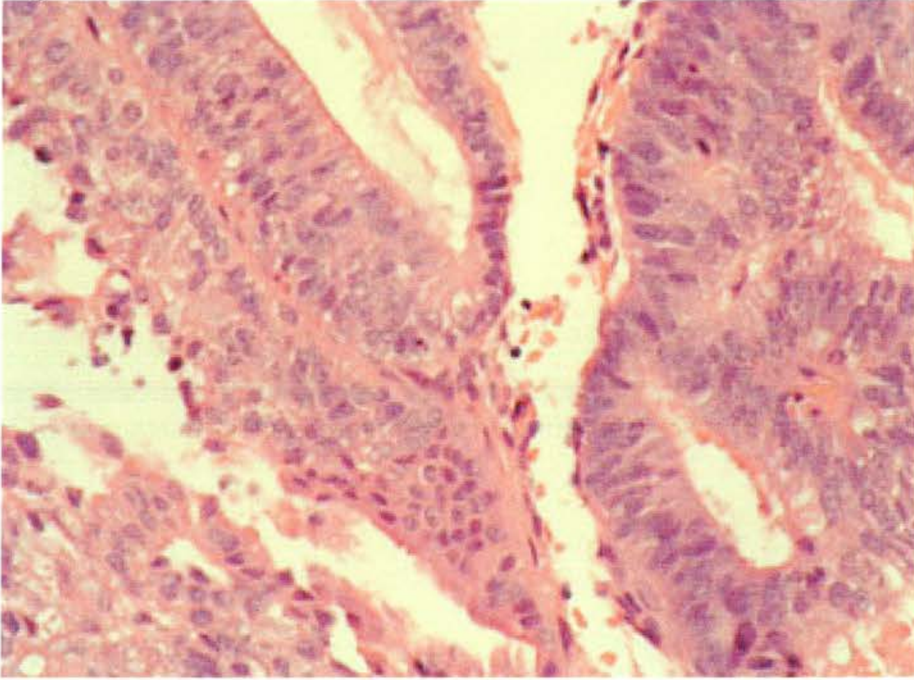


Figure 2.8

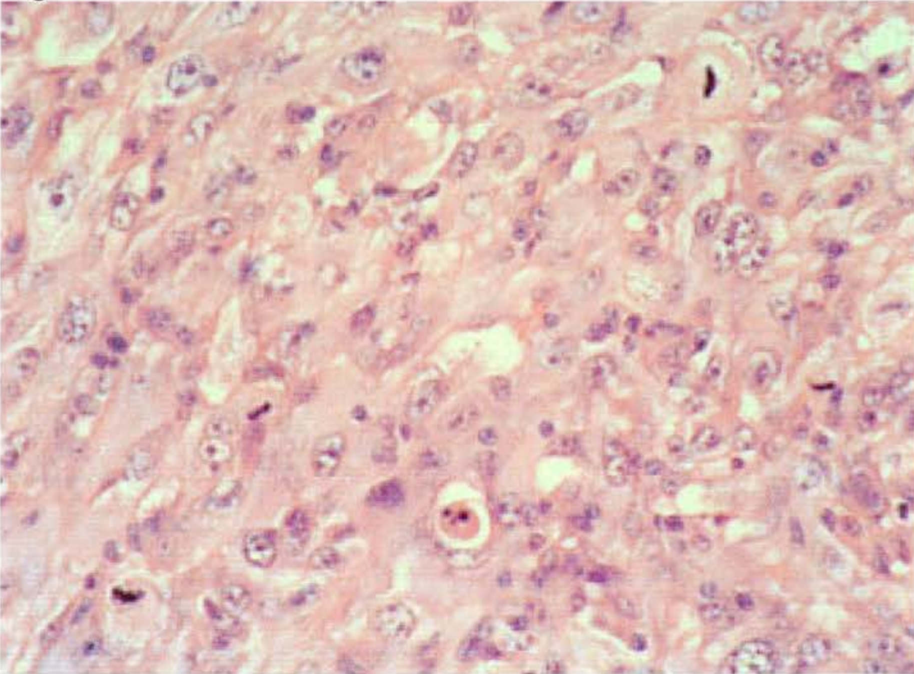


Figure 2.7. Stratification of cells in glands in Grade 1 EEC (H&E x20).

Figure 2.8. Pleomorphic nuclei in Grade 3 EEC (H&E x10).

Figure 2.9

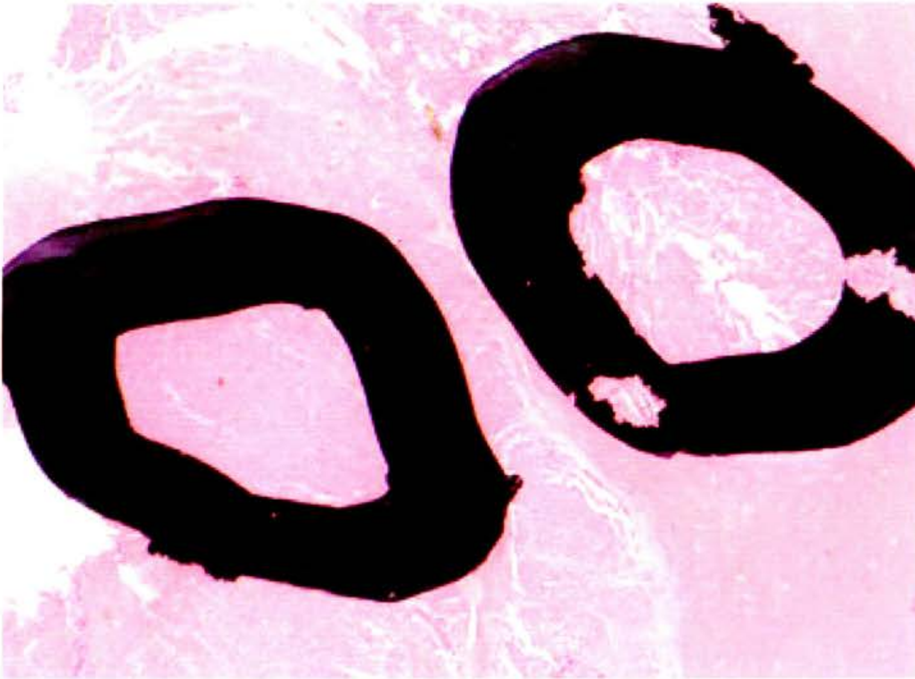


Figure 2.9. Circles showing area to be sampled by Beecher Instrument. At least three cores can be taken from within each circle (Magnificationx4).

Table 2.3 Grading of Endometrial Carcinoma.

	Grade 1 (Well Differentiated)	Grade 2 (Moderately Differentiated)	Grade 3 (Poorly Differentiated)
Percentage of Glands	>95%	>50%	<50%
Percentage of Solid Growth	<5%	<50%	>50%
Nuclear atypia	Increase grade by 1.		

2.2 Tissue Microarray

A tissue microarray (TMA) instrument by Beecher Instruments was selected (Figure 2.10). In order to construct the microarray, empty paraffin blocks of a depth 5-10mm were produced. Cores of wax, 0.8mm diameter were extracted from the empty blocks and replaced with cores of 0.6mm diameter taken from the tissue blocks at sites corresponding to the previously selected areas on the H&E slides (Figure 2.11). Two cores were taken from each area marked on the slide, so that at least two cores were taken from each case. The cores were punched at 1mm intervals, at least 2 cores per case, and the numbers of cores taken from each case ranged from 2 to 10. A grid system with each core having a coordinate reference (X axis, Y axis) was used to allow cross-reference between core location and parent case.

Once the TMAs were complete the blocks were sealed in a 60^0C oven for 10 minutes.

Figure 2.10

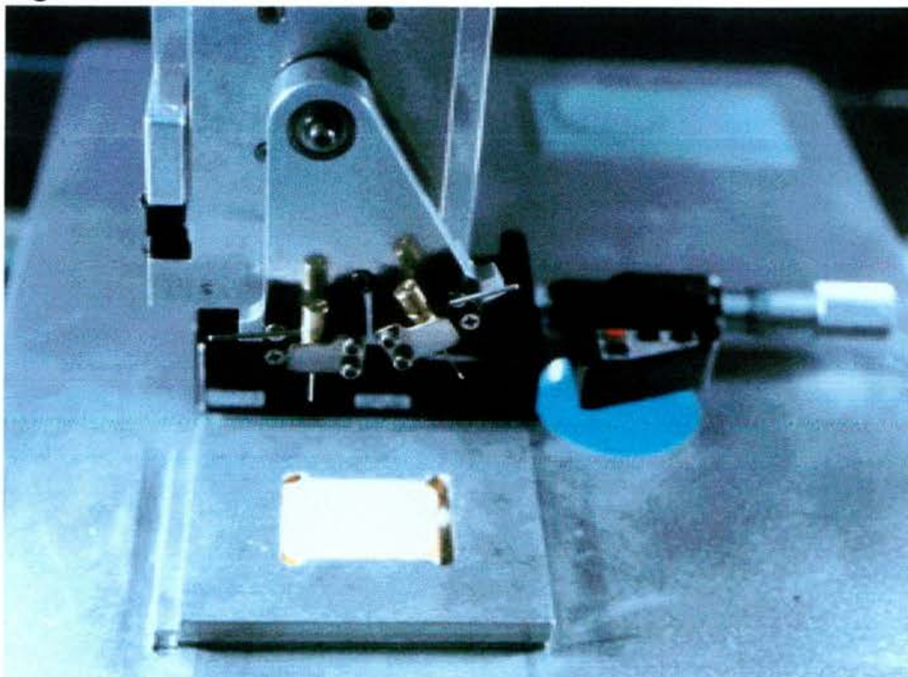


Figure 2.11

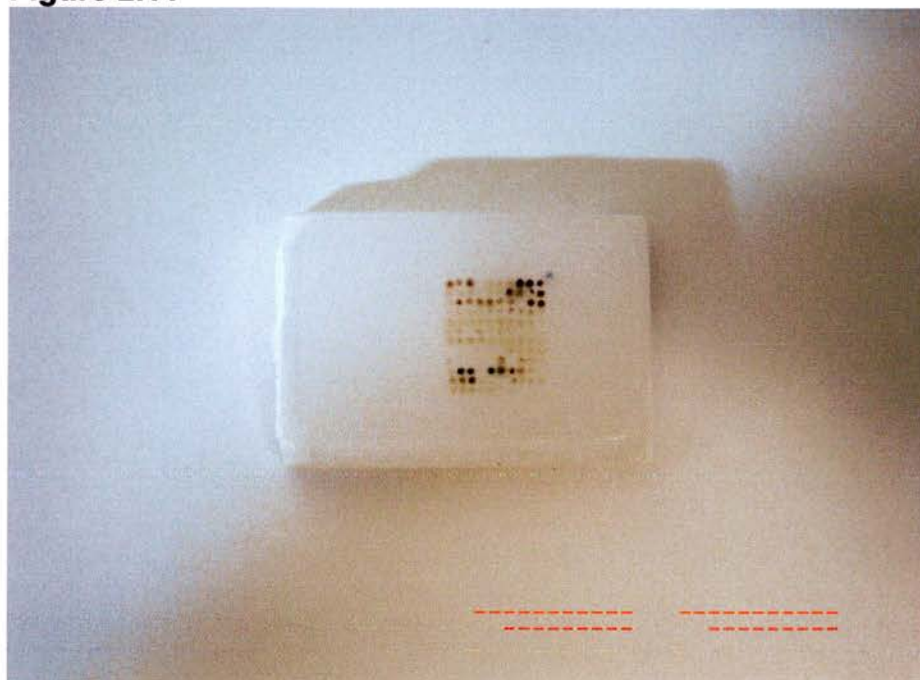


Figure 2.10. Beecher instrument used to create Tissue Microarrays courtesy of Skacel et al.

Figure 2.11. Tissue Microarray Block used for EEC cases.

2.3 Immunohistochemistry

From the TMA blocks, sections were cut at 3μm thickness and mounted on positively charged capillary action slides (Dako, Copenhagen, Denmark) and incubated at 60 ^{0}C overnight. The slides were dewaxed and rehydrated prior to antigen retrieval. Table 2.4 shows a summary of the details of the antibodies used.

2.3.1 P53

Antigen retrieval was performed by immersing the test slides and a case of breast carcinoma (as the positive control) in 0.01M ethylenediamine tetraacetic acid (EDTA), PH8.0 and microwaving at high power for 15 minutes. The primary antibody was the D-07 clone (Dako, Denmark) and was used at dilution 1:50. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.2 ER

Antigen retrieval was performed by immersing the test slides along with a slide of breast carcinoma (as the positive control) in 0.01M EDTA, PH8.0 and microwaving at high power for 15 minutes. The primary monoclonal antibody was the 6F11/2 clone (Novocastra, UK) and was used at dilution 1:200. Negative controls were obtained by omitting the primary antibody. All slides were stained on the on the Dako TechMate™ 500 plus, using the menapath supersensitive detection kit polymer HRP and the standard operating procedure for routine immunohistochemistry.

Table 2.4. Primary Antibodies and Antigen Retrieval Conditions used in IHC.

Antigen	Manufacturer	Clone	Dilution	Antigen Retrieval	Control
P53	Dako, Denmark	D-07	1:50	EDTA Microwave	Breast Carcinoma
ER	Novocastra Laboratories, UK	6F11/2	1:200	EDTA Microwave	Breast Carcinoma
MMP-2	Novocastra Laboratories, UK	17B11	1:40	EDTA Microwave	Appendix
MMP-7	Chemicon International, UK	1D1	1:25	None	Breast Carcinoma
MMP-9	Novocastra Laboratories, UK	15W2	1:80	EDTA Pressure Cooker	Kidney
PR	Dako, Denmark	PgR 636	1:100	EDTA Microwave	Breast Carcinoma
Galectin 3	Novocastra Laboratories, UK	9C4	1:150	EDTA Microwave	Papillary Carcinoma of Thyroid
CD98	Santa Cruz	Polyclonal IgG	1:200	Pressure cooker Microwave	Tonsil
E Cadherin	Dako, Denmark	NCH-38	1:25	EDTA Pressure Cooker	Tonsil
P Cadherin	Novocastra Laboratories, UK	56C1	1:100	EDTA Microwave	Placenta
β Catenin	Dako, Denmark	β catenin-1	1:50	EDTA Microwave	Tonsil

2.3.3 MMP-2

Antigen retrieval was performed by immersing the test slides along with a section of normal appendix (as the positive control) in 0.01M EDTA, PH8.0 and microwaving at high power for 15 minutes. The primary monoclonal antibody was the 17B11

clone (Novocastra Laboratories, UK) and was used at dilution 1:40. Negative controls were obtained by omitting the primary antibody.

All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.4 MMP-7

No antigen retrieval was performed. A slide of breast carcinoma was used as the positive control. The primary monoclonal antibody was the clone 1D2 (Chemicon International, UK) and was used at dilution 1:25. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.5 MMP-9

Antigen retrieval was performed by immersing the test slides along with a slide of kidney (as the positive control) in 0.01M EDTA, PH8.0 and pressure-cooking for 7 minutes. The primary monoclonal antibody was the clone 15W2 (Novocastra Laboratories, UK) and was used at dilution 1:80. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.6 PR

Antigen retrieval was performed by immersing the test slides along with a slide of breast carcinoma (as the positive control) in 0.01M EDTA, PH8.0 and microwaving at high power for 15 minutes. The primary monoclonal antibody was the PgR 636 clone (Dako, Denmark) and was used at dilution 1:100. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.7 Galectin-3

Antigen retrieval was performed by immersing the test slides along with a slide of papillary carcinoma of the thyroid (as the positive control) in 0.01M EDTA, PH8.0 and microwaving at high power for 15 minutes. The primary monoclonal antibody was the 9C4 clone (Novocastra, UK) and was used at dilution 1:150. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.8 CD98

Antigen retrieval was performed by immersing the test slides along with a slide of tonsil (as the positive control) in PC citrate and microwaving at high power for 2.5 minutes. The primary polyclonal IgG antibody was the clone C-20 (Santa Cruz) and was used at dilution 1:200. A rabbit antigoat secondary antibody was used. Negative controls were obtained by omitting the primary antibody. All slides were stained on

the Dako TechMate™ 500 plus, using the ABC-vector kit and the standard operating procedure for routine immunohistochemistry.

2.3.9 E Cadherin

Antigen retrieval was performed by immersing the test slides along with a slide of tonsil (as the positive control) in 0.01M EDTA, PH8.0 and microwaving at high power for 2.5 minutes. The primary monoclonal antibody was the NCH-38 clone (Dako, Denmark) and was used at dilution 1:25. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.10 P Cadherin

Antigen retrieval was performed by immersing the test slides along with a slide of placenta (as the positive control) in 0.01M EDTA, PH8.0 and microwaving at high power for 15 minutes. The primary monoclonal antibody was the 56C1 clone (Novocastra, UK) and was used at dilution 1:100. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.3.11 β Catenin

Antigen retrieval was performed by immersing the test slides along with a slide of tonsil (as the positive control) in 0.01M EDTA, PH8.0 and microwaving at high

power for 15 minutes. The primary monoclonal antibody was the β catenin-1 clone (Dako, Denmark) and was used at dilution 1:50. Negative controls were obtained by omitting the primary antibody. All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

2.4 Scoring

To score P53, a similar scoring system to that used by Leversha et al was used {Leversha, 2003}. As all cores showed homogenous staining, the intensity was scored. The intensity was classified into 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining. The positive control was used as a comparator for strong staining. As the staining pattern is known to be homogenous, the maximum score of P53 was used in calculations.

ER and PR were scored using a histoscore used in assessing expression in breast carcinoma cases. This was created by adding the intensity of staining (where 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining) to the percentage of cells stained (where 0 = no cells staining, 1 = 1-9% of cells staining, 2 = 10-33% cells staining, 3 = 33% - 66% cells staining, 4 = 67-99% cells staining and 5 = 100% cells staining). This gave a range of scores from 0 to 8. As both ER and PR show heterogeneous staining and both had a wide range of possible scores, the average score of the informative punch biopsies was used.

The scores for MMP-2, MMP-7, MMP-9, Galectin-3, CD98, E cadherin, P cadherin and β catenin were assessed using a histoscore. This was calculated by multiplying the intensity of staining (1=weak, 2=moderate and 3=strong) by the percentage of

cells showing positive staining (1=<20%, 2= 20-80% and 3=>80%), thus giving a score between 1 and 9. To minimise erroneous results, the average score between the sample cores' score was used in calculations.

As there is some evidence that the site of staining of Galectin-3 changes according to action of Galectin-3, the site of staining, either cytoplasmic, nuclear or cytoplasmic and nuclear was noted.

As we were expecting a decrease in E cadherin expression compared to normal endometrium, the fragments of normal endometrium within the TMAs were used as a positive control, as well as the section of tonsil. All the fragments of endometrium and epithelium in the section of tonsil showed strong, universal expression, with an overall score of 9.

To ensure the scoring was consistent, the TMAs stained for E cadherin, P cadherin, β catenin and ER were rescored 6 months after the initial scoring. Kappa statistics were applied to the repeat scores and showed good correlation with the results with a score of 0.63.

2.5 Statistical Analysis

All statistical analysis was performed using SPSS 13.0 software (SPSS Inc, Chicago).

2.5.1 Tissue Microarray

The mean scores of the representative cores of tumour assessed for each case of USPC and EEC were calculated. The results from the main part of the tumour were used in calculations to determine if there were any differences in expression of

antibodies between tumour types. A paired T-test was performed for the parametric results and a Mann Whitney U test was performed for the non-parametric results. As multiple comparisons were being made, there is a risk that this could be interpreted as "a fishing expedition" and erroneous significant differences may be detected. Therefore a Bonferroni correction was made. This is a statistical adjustment of the p value for multiple comparisons, decreasing the liklehood of erroneous false positive findings. Therefore, for Chapters 5 and7, a p value of less than 0.017 was considered significant and for Chapter 6, a p value of less than 0.025 considered significant.

The Wilcoxon matched-pairs signed-ranks test was used to estimate relationship between staining patterns of the different antibodies used between the main part of the tumour and at the invasive edge. A p value of less than 0.05 was considered significant. The Pearson pairwise correlation coefficient was used to determine any relationship between the antibody expressions.

2.5.2 Audit of the Pathology and Management of Uterine Papillary Serous Carcinoma (UPSC) and Correlation with Outcome (Chapter 4)

The Spearman's Rank test was used to assess the correlation of the initial diagnostic biopsy with the definitive hysterectomy histology percentage histological subtype. Overall survival and progression free survival were estimated on the basis of Kaplan-Meier curves and the log-rank test used as a univariate analysis of prognostic factors. A p value of less than 0.05 was considered statistically significant, and variables that had a p value of less than 0.05 were entered into a Cox regression model.

Chapter 3

Validation of Tissue Microarray of Endometrial Carcinoma

3.1. Introduction

Battifora et al {Battifora, 1986} introduced the multitumour tissue block in 1986 and Kononen et al {Kononen, 1998} refined this technique to develop the tissue microarray (TMA) in 1998. Since then the TMA has been widely used in retrospective analysis of large numbers of formalin fixed paraffin embedded tumours. The use of TMAs allows screening of up to several hundred tumours for immunohistochemistry (IHC) or fluorescent *in situ* hybridisation (FISH) on a single block, with a minimum outlay of reagents. As all the cores are of the same size and because all the cores are present on limited numbers of slides they can be treated identically with regards to antigen retrieval and staining thus reducing the experimental errors and discrepancies produced during analysis.

A potential shortcoming of TMA is sampling bias, whereby lack of tumour representation in the cores selected may give erroneous results, due to heterogeneity of tumours. To address this issue, the sampling of multiple cores is recommended from each case. However, there is uncertainty as to the optimal number of cores that need to be taken to give a result that is representative of the tumour as a whole. As this is likely to vary with different tumour types, it is advisable to perform a validation study from tumours arising in specific organs. Validation studies have been performed and published on a range of tumours including those from the breast, prostate, gastrointestinal tract and ovary {Gillett, 2000;Mousses, 2002;Gulmann, 2003;Rosen, 2004},and have recommended between 2 and 6 cores be taken from

each case. No tissue validation study on endometrial carcinoma has yet been published in the literature, therefore a validation study was performed to determine whether TMAs could be used in place of whole sections for IHC and, in addition, to calculate the number of cores needed from each case to ensure adequate representation of the tumour.

3.2 Materials and Methods

3.2.1 Tissues

Archival tissue samples of 78 cases of Uterine Papillary Serous Carcinoma (UPSC) were selected from the Royal Infirmary of Edinburgh Department of Pathology, the slides were reviewed by myself and Dr A Williams (a subspecialist gynaecological pathologist), the diagnosis of UPSC was confirmed, using the criteria described in "Materials and Methods", and the appropriate blocks were selected. The H&E sections then had at least one area from the centre of the tumour marked on the slide by the pathologist. All tissue had been routinely fixed in 4% buffered formaldehyde and processed into paraffin blocks. Forty-three of the 78 tissue blocks used in the TMA also had immunohistochemistry (IHC) performed on whole-tissue sections for comparison of the immunohistochemical staining patterns.

3.2.3. Tissue Microarray

The TMA was created using the technique as described in " Material and Methods". Once the TMA was made and sealed, sections were cut, with the most superficial section being stained with Haematoxylin and Eosin (H&E) and the deepest being used for IHC for Oestrogen Receptor (ER) and P53.

3.2.4. Immunohistochemistry

Both ER status and P53 expression has been well documented in UPSC {Demopoulos, 1999;Vasil'eva, 2005;Wu, 2003}. ER expression is known to be lower in UPSC than in endometrioid endometrial carcinoma (EEC) and P53 expression is increased in UPSC compared with EEC {Demopoulos, 1999}. In addition ER expression tends to be heterogeneous whereas P53 expression tends to be homogenous. Therefore antibodies to ER and P53 were used to validate the TMA,

using the techniques described in “Materials and Methods”. Expression of both antibodies was assessed using the systems described in “Materials and Methods”.

3.3 Results

3.3.1 Technical Efficiency of Tissue Microarray Construction

Technical issues, including poor sealing may result in poor section quality and misalignment of cores. Misalignment of cores can result in higher cores being cut through and disappearing before other cores placed lower in the block emerge.

The H&E, P53 and ER sections were screened and the presence of tumour was checked at each level. Table 3.1 shows a summary of the efficiency of the sections taken from the TMA. Fourteen percent of cores on the H&E section did not show tumour and were not informative. Of these, 8 cores showed blood only, 19 showed myometrium only and 19 cores were missing. This resulted in 2 tumours not being represented by the TMA. A similar result was seen with the P53 slide with 8 cores showing blood only, 19 showed myometrium only and 23 cores were missing. This resulted in 4 cases not being represented by the TMA. Six of the ER cores showed blood only, 17 showed myometrium only and 22 cores were missing. This resulted in 8 cases not being represented.

3.3.2 Technical Aspects of TMA Immunohistochemistry

The percentage of informative cores was 86%, 85% and 86% for H&E, P53 and ER respectively. Although there was no significant increase in core loss as the sections were taken through the TMA block, the number of cases not represented increased through the TMA. This appeared to be due to different cores cutting out the tumour at deeper levels resulting in tumour loss. Appendices 2 and 3 show the individual results of each core compared to the whole sections.

All P53 staining was nuclear and appeared homogeneous (Figure 3.1). Table 3.2 shows the results of the sections stained with P53. Of the 74 cases with informative cores stained for P53, 54 showed complete agreement of scores between cores. In 14 cases there was a difference in score of 1, 3 cases had a difference in score of 2 and one case showed a difference of 3 in the scoring (where 1 punch was negative, 3 showed a score of 2 and 4 showed a score of 3). Counting the score +/- 1 as concordance, this gave an overall concordance of 92% between cores. When the scores were compressed into positive and negative the overall concordance between cores was 93%.

All ER staining was nuclear and in some cases appeared heterogeneous (Figure 3.2). Table 3.2 shows the results of the ER IHC. Of the 70 cases stained with ER with informative punches, 41 either had the same score or a difference of 1 between all punches in the same case, 15 cases had a difference of 2, 6 cases had a difference of 6 and 9 cases had a difference in scores of more than 4. This gave an absolute concordance of 59%. When the ER scores were compressed into positive (greater than 4) or negative (less than 4), 58 cases had the same score and in 13 cases, the variation in scores between punches ranged from positive to negative. This gave an overall concordance of 82%.

Figure 3.1

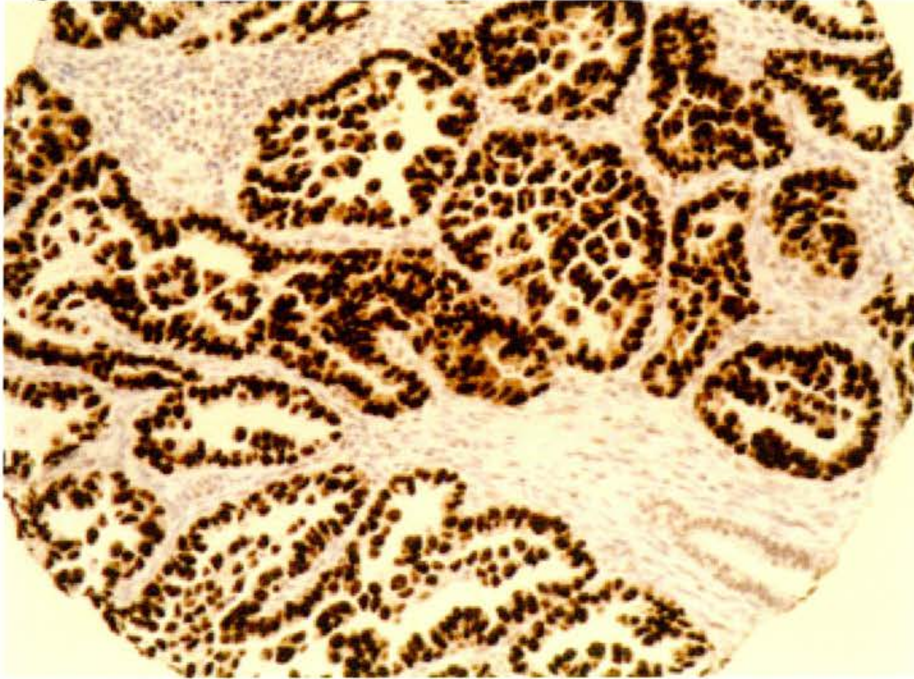


Figure 3.2

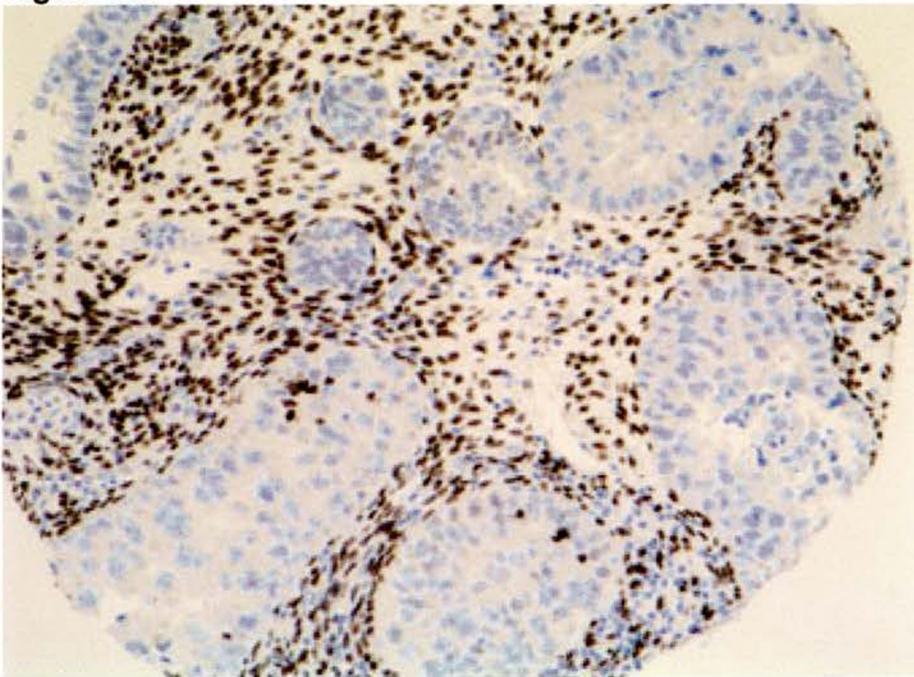


Figure 3.1. P53 Immunohistochemistry in UPSC Tissue Microarray (Magnificationx20).

Figure 3.2. ER Immunohistochemistry in UPSC Tissue Microarray (Magnificationx20).

Table 3.1 Technical Results of TMA stained with H&E, P53 and ER

Section	Tumour present	Tumour not present	Total number of cases	Informative cores	Non Informative Cores			Total number of cores
					Blood	Myometrium	Core Missing	
H&E	76	2	78	283	8	19	19	329
P53	74	4	78	279	8	19	23	329
ER	70	8	78	284	6	17	22	329

3.3.3 Comparison of Data from Whole Sections and Microarray Samples

3.3.3.1 P53

Expression of P53 in the whole sections was similar to that seen in the TMA (Figure 3.3). Table 3.2 shows the results of the whole sections stained with P53. When the consensus result for the replicate punches was compared with the IHC result from the whole sections, the data were concordant for 35 of 39 tumours (90%) for P53. In the four discordant cases the whole sections results were higher compared with the TMA results. When the data were compressed into positive and negative, 34 of the 39 tumours gave the same result, with an 87% concordance. In the 5 discordant results, in all cases, the whole sections were positive and the TMA results were negative.

Simple regression was used to determine whether the compressed or non-compressed TMA P53 score could predict the P53 score on whole sections. In both cases this showed a correlation (R) of 0.814 and the coefficient of determination (r^2) of 0.663. This is a large effect size and indicated that the TMA P53 score was a good predictor of the P53 score on whole sections.

3.3.3.2 ER

On examining the whole sections, most tumours revealed heterogeneous nuclear staining for the ER antibody (Figure 3.4). This was more apparent than on examination of the TMA sections. Table 3.2 shows the results of the whole sections scored with ER. The assessment of concordance was more difficult between the TMA and whole section for ER as scores ranged from 0 to 8. However in comparing the whole section result with the TMA score, a result that was +/- 1 was deemed to

be concordant. Therefore when the consensus result for the replicate punches was compared with the IHC result from the whole sections, the data were concordant for 22 of 36 tumours (61%) for ER. However, when the consensus data was distributed into positive and negative we showed that 31 of 36 cases (86%) had absolute concordance with the whole sections and in the 5 discordant cases the whole sections were positive and the TMA sections were negative (false negative results). There were no false positive results.

Simple regression was used to determine whether the TMA ER score could predict the ER score on whole sections. This showed a correlation (R) of 0.727 and the coefficient of determination (r^2) of 0.515. This was a large effect size and indicated that the TMA ER score was a good predictor of the ER score on whole sections. When the ER results were compressed into positive and negative the correlation (R) was 0.756 and the coefficient of determination was 0.559. This again, was a large effect size and indicated that the TMA ER score was a good predictor of the ER score on whole sections.

Figure 3.3

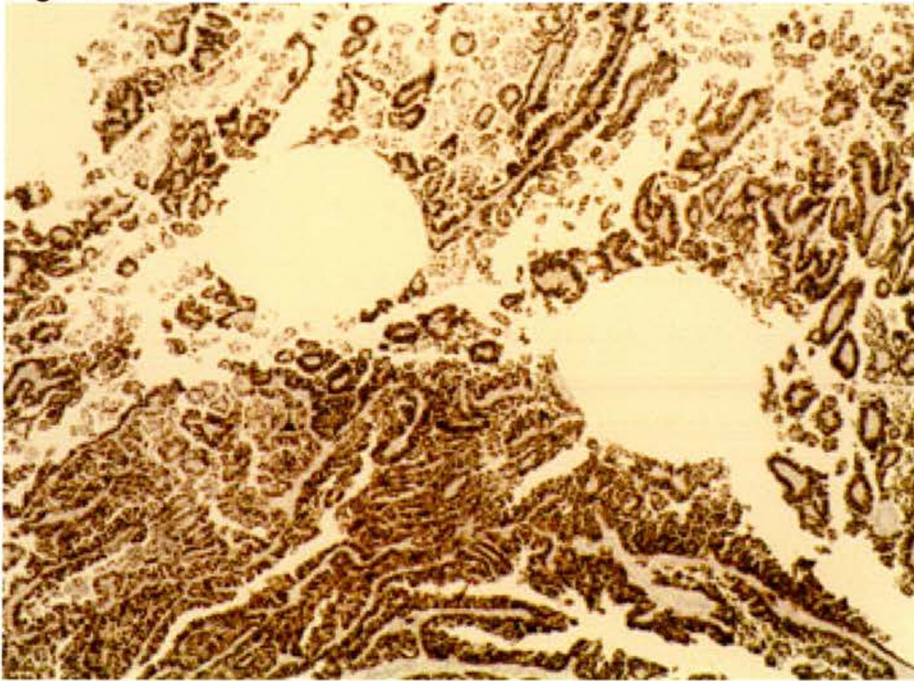


Figure 3.4

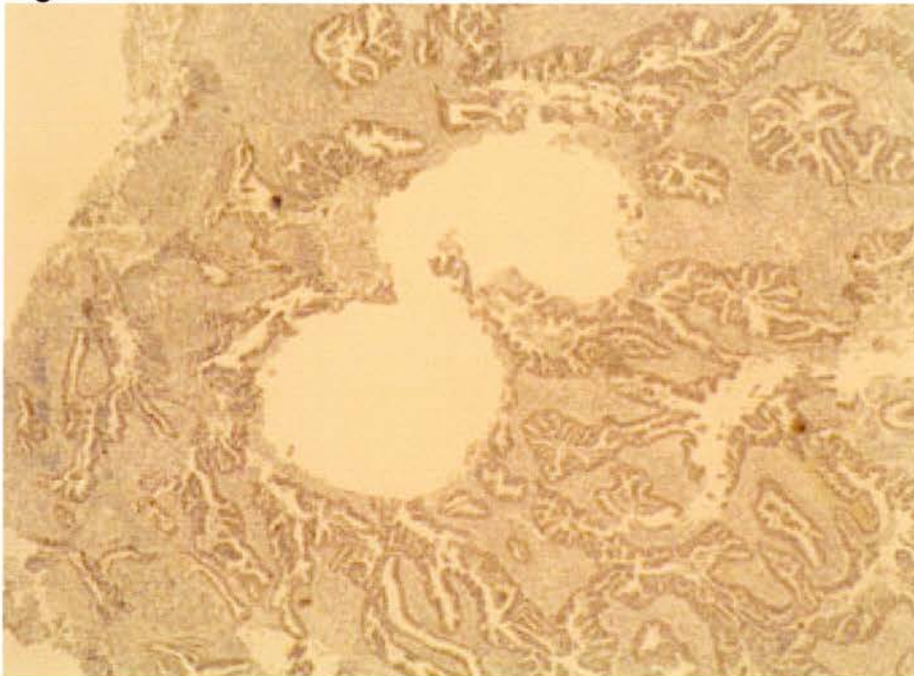


Figure 3.3. Whole section showing P53 Immunohistochemistry with hole at the Site of Core Biopsies (Magnificationx20).

Figure 3.4. Whole section showing ER Immunohistochemistry with hole at the Site of Core Biopsies (Magnificationx20).

Table 3.2 P53 and ER Expression in TMA and Whole Sections

	P53		ER	
	Positive	Negative	Positive	Negative
TMA Total number = 68	56 (82%)	12 (18%)	39 (57%)	29 (43%)
Whole Sections Total number = 42	40 (95%)	2 (5%)	23 (54%)	19 (46%)

3.4 Discussion

The objective of this chapter was to determine whether TMAs of UPSC could be used for IHC in place of whole sections. P53 and ER expression in TMAs was concordant with that of whole sections and the levels of expression in both TMAs and whole sections were generally in agreement with that in the literature {Demopoulos, 1999;Wu, 2003;Vasil'eva, 2005}. Agreement was higher with P53 where expression appeared more homogenous compared with ER where expression tended to be heterogeneous. It is possible that the different scoring criteria applied, with 4 categories for P53 and 8 categories for ER, also affected the level of concordance.

Leversha et al {Leversha, 2003} suggested that the most robust method of assessing antibody expression was to use 2 categories, either "positive" or " negative", whereas Gomaa et al {Gomaa, 2005} recommended splitting results into 4 groups for analysis. However, simplifying the scoring can produce erroneous results when the antibody staining pattern is heterogeneous. Compressing the ER results into positive and negative improved concordance, but there was no improvement in concordance when the P53 results were compressed. This may be due to the wider possible range of scores available for ER, whereas P53 only had 4 categories. Therefore, I feel that, where the antibody staining pattern is uncertain it is wiser to adopt a Histoscore-type scoring system, which gives a wider range of categories and thus may minimise erroneous results.

Reviewing previous validation studies, the statistical techniques used in evaluating validation have varied and have included kappa analysis, regression analysis, Pearson correlation coefficient, and re-sampling methods {Hoos, 2001;Gillett,

2000;Rosen, 2004;Rubin, 2002}. I used simple regression analysis as I felt this most directly answered the research question "can TMAs be used to predict the pattern of expression of an antibody from whole sections". For both P53 and ER we demonstrated a large effect size supporting the conclusion that TMAs can be used in place of whole sections.

The second objective was to determine the optimal number of cores that needed to be taken from each case to give the most accurate results. Our informative yield was approximately 86% in sections taken from both the top and bottom of the TMA block. Punch loss occurred in 6%, 7% and 7% of sections stained with H&E, P53 and ER respectively and this is similar to that in the established literature {Leversha, 2003;Wu, 2003;Demopoulos, 1999}. In all sections taken the majority of uninformative punches were due to sampling error where all that was present was either myometrium or blood. The punches showing myometrium only may reflect that some of the samples were taken from Stage Ia UPSC (Figure 3.5) and therefore the tumour was focal and was cut through in deeper sections or may have been taken from cases where the tumour spread in an infiltrative pattern rather than as a solid mass (Figure 3.6), thus making sampling more difficult. The punches showing blood only were cases where the tissue sampled were endometrial pipelle specimens, where often scanty fragments of tumour lie in blood and therefore these punches were prone to being cut through and the tumour was more susceptible to being missed during sampling. Overall our percentage of uninformative samples was at the higher end of the range reported in the literature (5-17%) {Leversha, 2003;Rosen, 2004;Fernebro, 2002;Hoos, 2001;Gillett, 2000}, but our punch loss was similar (6-7% compared

Figure 3.5

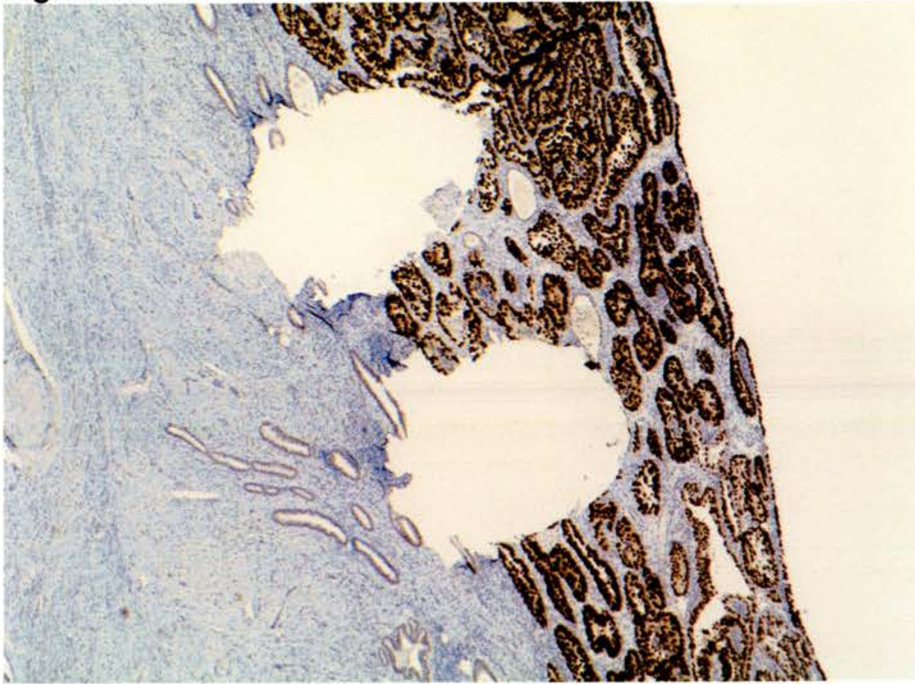


Figure 3.6

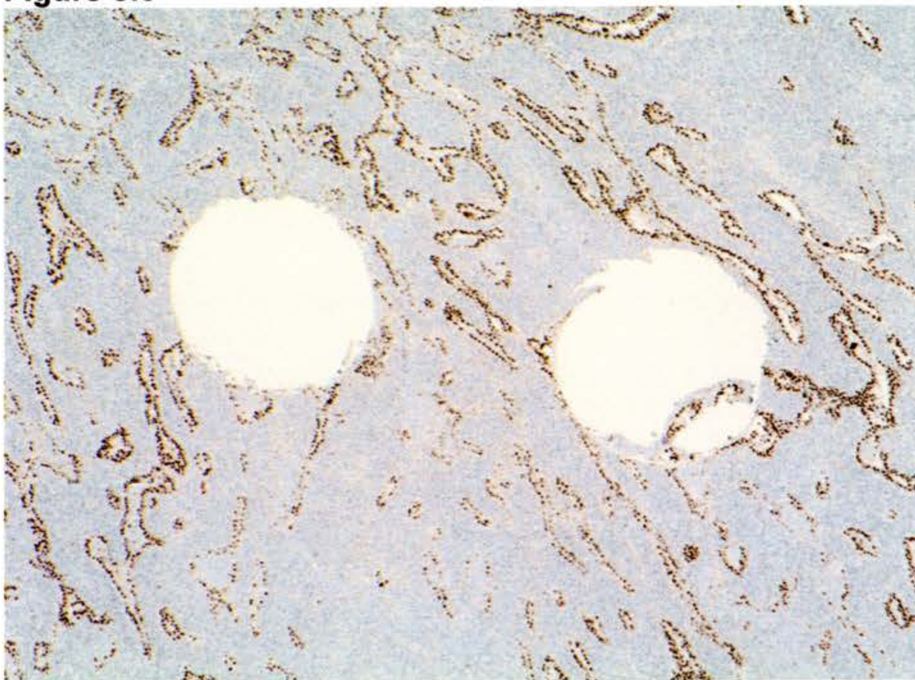


Figure 3.5. Whole Section Showing P53 Staining and Site of TMA cores in Stage 1a UPSC (Magnificationx4).

Figure 3.6. Whole Section Showing P53 Staining and Site of TMA cores in infiltrating UPSC (Magnificationx4).

with 5% {Leversha, 2003}. The difference in informative punches in part reflects the diversity of specimens being sampled, rather than solid fragments of tumour, which are most commonly used in creating TMAs.

We found that taking more than one punch from each case increased the level of informative data from the TMA, which otherwise would have been lost. In addition, in the case of ER where expression was heterogeneous this lessened discrepancies. It is difficult to determine the exact number of punches, which should be taken from each case to ensure adequate and accurate representation, but in the case of the pipelle specimens more specimens were advisable. Torhorst et al {Torhorst, 2001} suggested that 4 cores was optimal for measuring Progesterone Receptor expression, but that only one core was necessary in assessing prognostic significance when the data covered more than 390 patients. However, most validation studies have shown that analysis of three 0.6mm cores produces higher concordance rates than use of one core and that the use of three cores also reduces the problems associated with case loss where only 2 cores are taken {Camp, 2000;Kallioniemi, 2001;Fernebro, 2002}.

Studies have shown difficulties assessing the significance of protein expressed at a low level in TMA and whole sections {Leversha, 2003}. However I did not find this to be the case where ER expression was not only low but also heterogeneous.

Whilst the UPSC TMA was validated for P53 and ER the obvious question is whether or not this validation can be extended to include all high grade endometrial carcinomas and also to the assessment of antibodies where the normal or abnormal staining patterns are uncertain. As we have demonstrated good levels of concordance with ER and P53 which not only are known to be present in low and high amounts respectively but also show heterogeneous and homogenous patterns of staining

respectively {Koshiyama, 1995}, the conclusion I have drawn is that the validation results can be extended to the wider group of endometrial carcinomas and unknown antibodies.

3.4.1 Summary

The protein expression of P53 and ER in our TMA matched that seen in the whole sections. On balance the minimum number of cores sampled should be two and the optimal number of cores sampled should be three as this decreases variation seen in heterogeneous tumours and also increases the likelihood of an informative section in those tumours which were either focal, infiltrative or present in a pipelle specimen. In addition TMA use can be extended to include other high grade endometrial carcinomas and antibodies with uncertain patterns of expression.

Chapter 4

A Review of the Pathology and Management of Uterine Papillary Serous Carcinoma and Correlation with Outcome

4.1 Introduction

While papillary morphology has been recognised in endometrial carcinoma since the turn of the last century, uterine papillary serous carcinoma (UPSC) was first described as a distinct clinical entity in 1982 by Lauchlan {Lauchlan, 1981} and Hendrickson et al {Hendrickson, 1982;Hendrickson, 1982} UPSC usually occurs in elderly, postmenopausal women and in contrast to endometrioid endometrial carcinoma (EEC), is not associated with excess oestrogen. There is conflicting data regarding the association of breast cancer with high-risk endometrial carcinomas {Barakat, 1994;Magriples, 1993}. It is thought that patients with breast cancer have a similar rate of low- and high-risk endometrial subtypes irrespective of tamoxifen use. However, patients with UPSC may have an increased risk of synchronous, or subsequent development of breast cancer {Geisler, 2001;Goshen, 2000}. This raises the possibility that UPSC and breast cancer may, in some patients, be due to the presence of mutations in cancer predisposing genes such as BRCA 1, BRCA2 or p53 {Lavie, 2004}.

Although accounting for only 10% of endometrial carcinomas, UPSC is an important diagnosis to make on initial biopsy since it behaves particularly aggressively, with a propensity for early metastasis which results in upstaging at the time of operation in 50-75% of clinical Stage I cancers {Goff, 1994;Christman, 1987;Walker, 1982}.

This underscores the need for accurate pre-operative diagnosis, so that the surgeon can plan definitive treatment and perform accurate and thorough surgical staging by the FIGO system, including a total extrafascial hysterectomy and bilateral salpingo-oophorectomy, peritoneal washings, and removal of any suspicious pelvic or para-aortic lymph nodes. Although omentectomy is not in the 1988 FIGO system for staging, it is also frequently performed. This is because UPSC behaves aggressively, and has a pattern of spread similar to that of ovarian serous carcinoma. There is no evidence to date that this radical surgery affects outcome, but accurate staging may help influence current advice regarding adjuvant therapy. UPSC may exist in both 'pure' and 'mixed' forms with other endometrial cancer subtypes, in particular EEC and clear cell endometrial carcinoma (CCEC). Sherman et al {Sherman, 1992} states that the diagnosis of UPSC should be reserved for cancers with more than 25% UPSC pattern in the final resection specimen, although this figure appears arbitrary.

Data as to the optimum management of UPSC are limited due to its relative rarity. This audit was performed to look at the patient characteristics, histopathological characteristics and outcomes of various treatment regimens of UPSC. In particular, this study focussed on the accuracy of diagnosing UPSC on endometrial pipelle biopsies or curettings, whether the histological subtypes seen in the diagnostic biopsy correlated with those seen in the final resection specimen, and also what percentage of UPSC pattern was required to confer the poor prognostic phenotype. To date there have been several studies looking at either the oncological or surgical management of UPSC, ranging from 9 to 129 cases {Abeler, 1990;Carcangiu, 1992;Chambers,

1987;Christopherson, 1982;Geisler, 2001;Goff, 1994;Hendrickson, 1982;Slomovitz, 2003;Sykiotis, 2001}.

4.2 Materials and Methods.

4.2.1 Identification of Cases.

Cases were identified as described in "Materials and Methods".

The patient demographics, histopathology, and treatment were recorded as described in "Materials and Methods".

4.2.2 Patient Characteristics.

Surgical and oncological medical records were reviewed by Dr A Stillie (an Oncology Specialist Registrar) to obtain patients' age at presentation, any previous history of breast cancer, the treatment and follow-up data, in particular the site of any relapse. The primary endpoints were progression-free survival (PFS) and overall survival (OS), defined as the period from the date of diagnosis to the date of recurrence or the last clinic visit (if alive) or the date of death. Data were censored if information on survival was not available or the patient had ceased to be followed up for any reason but had not died of cancer.

4.2.3 Histopathological Characteristics.

HM and Dr D Faratian (a Specialist Registrar in Pathology) reviewed all diagnostic biopsies and hysterectomy sections from the 67 patients. The diagnosis of UPSC, clear cell carcinoma (CCEC) and EEC were made using the criteria as described in "Material and Methods". The pipelle or endometrial curettings were reviewed to determine if definitive diagnosis of UPSC was possible on the original biopsy. Then the hysterectomy specimens were reviewed to determine if it was a pure UPSC or mixed tumour. If it was a mixed tumour, the percentage of UPSC, EEC and CCEC of the total tumour present was estimated in the tissue processed for histological analysis. Estimates were made by counting low power (x20-x40) fields of each

pattern and dividing this by the total number of fields. Data were compared and the three discrepancies resolved by discussion. The number and nature of specimens additional to the hysterectomy specimen received (i.e. omentum, pelvic lymph nodes or peritoneal washings) were noted, along with the pathological stage at the time of resection using the FIGO staging criteria as described in "Materials and Methods".

4.2.4 Oncological Characteristics

The adjuvant therapy was recorded and the chemotherapy and radiotherapy regimens (external beam +/- vaginal caesium) were documented. Where the data was available, details of the treatment of recurrence were also noted.

4.2.5 Statistical Analysis

Statistical analysis was performed as described in "Materials and Methods".

4.3 Results

4.3.1 Patient Characteristics

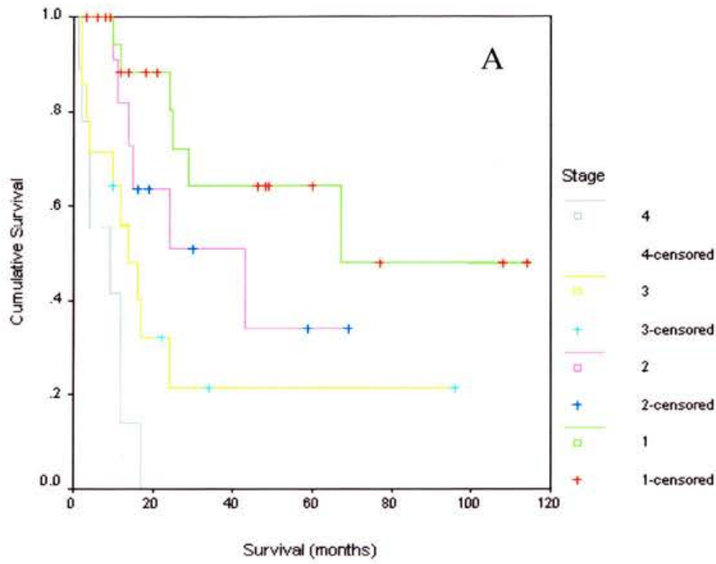
Sixty-seven patients were identified. The median age of the patients was 68 years (range 49-89). Nine patients (13%) had a past medical history of breast cancer. No patients developed breast cancer after the time of diagnosis of UPSC. The median follow-up for all patients was 14.5 months (range 1-114). The numbers and percentages of each patient with each stage of disease are presented in Table 4.1, along with 1 year and 3 year OS and PFS by stage. Overall survival and PFS correlated significantly with the stage of disease ($p<0.01$, Figure 4.1), the median OS was 67 months, 43 months, 14 months and 9 months for Stage I, II, III and IV disease, respectively. The median PFS for Stage I disease was not reached in this study, due to censoring, but the median PFS for Stage II, III and IV disease was 43 months, 9 months and 8 months respectively.

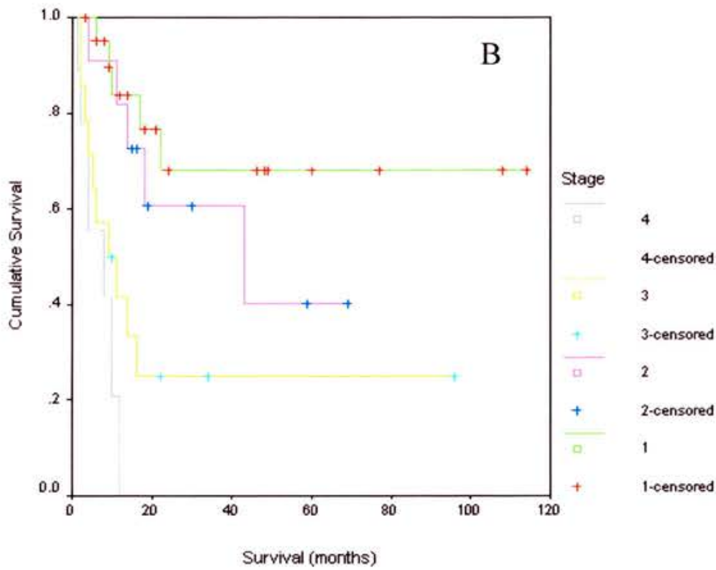
Table 4.1. Progression Free Survival and Overall Survival by Stage

Stage	Number of patients (%)	Progression Free Survival		Overall Survival	
		1 year	3 years	1 year	3 years
Stage I	25 (37)	84.7	*68.2	88.2	*64.1
IA	3 (4.5)				
IB	14 (21)				
IC	8 (12)				
Stage II	14 (20)	81.8	*60.1	81.8	*50.9
IIA	7 (10)				
IIB	7 (10)				
Stage III	19 (28)	41.7	*25.0	56.2	*21.4
IIIA	14 (21)				
IIIB	3 (4.5)				
IIIC	2 (3)				
Stage IV	9 (13)	0	ND	41.6	ND
All Patients	67	61.5	46.2	67.0	39.4

*PFS, $p<0.01$ (Kaplan-Meier); *OS, $p<0.01$ (Kaplan-Meier); ND = No data

Figure 4.1. Overall Survival and Progression-Free Survival by Stage.





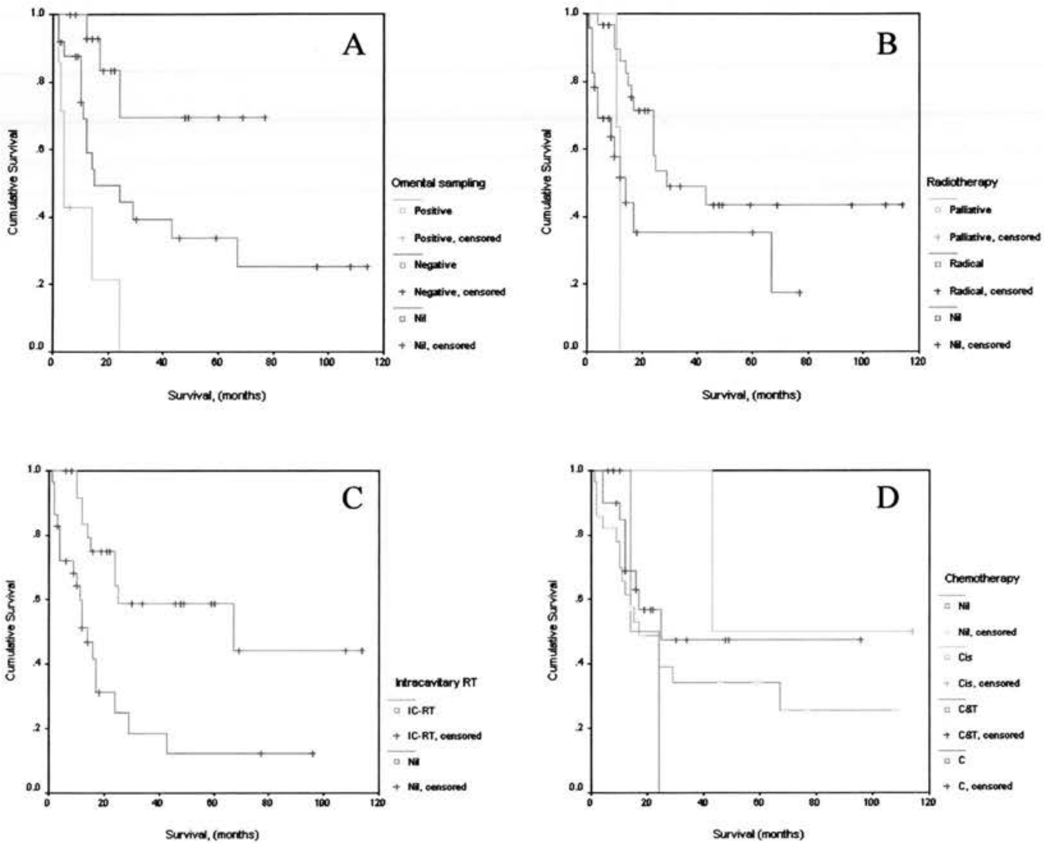
(A) Overall survival by Stage ($p<0.01$), (B) Progression-free survival by Stage ($p<0.01$).

4.3.2 Surgical Analysis

Fifty-nine patients underwent surgery. All these patients had a total abdominal hysterectomy, bilateral salpingo-oophorectomy (TAH BSO). Twenty-four patients (40.1%) had, in addition, omental sampling, of these, 21 patients (87.5%) an omental biopsy, where only a few centimetres of omentum was removed and 3 patients (12.5%) had full omentectomy, where all the visible omentum was removed. Seven patients (11.9%) had a pelvic node clearance; in 30 patients (50.9%) peritoneal washings were taken. Seven patients had TAH BSO, omentectomy, pelvic node sampling and peritoneal washings taken. No patients had para-aortic node sampling.

Seven (29%) omental samples were positive for metastatic disease, 2 (28.5%) pelvic lymph node clearances were positive, while 6 of 30 (20%) of peritoneal washings contained malignant cells. Patients who had a negative omental biopsy at the time of operation had a significantly better PFS and OS ($p<0.01$) than those patients who had a positive biopsy (Figure 4.2). Of the three omentectomies performed, one was positive and two were negative. Positive peritoneal cytology was weakly associated with poor prognosis (log-rank test, $p=0.15$), while the effect of pelvic lymphadenectomy on prognosis could not be analysed due to small numbers of patients.

Figure 4.2. Kaplan-Meier Curves of Treatment Variables by Overall Survival.



(A) Omental sampling $p<0.01$ (log-rank test), (B) Radiotherapy $p<0.01$ (log-rank test), (C) Intra-cavitary radiotherapy $p<0.01$ (log-rank test), (D) Chemotherapy $p=0.64$ (log-rank test).

Abbreviations: RT = radiotherapy, Cis = cisplatinum, C&T = carboplatin and paclitaxel (Taxol), C = carboplatin.

4.3.3 Histopathological Analysis

On reviewing the pathology reports, 63 of the cases were diagnosed by pipelle biopsy, curettage, or hysteroscopic biopsy; 1 case was diagnosed on cervical cytology, and 3 cases from hysterectomy histology. Of the 63 patients who had a pre-operative diagnostic biopsy, 23 biopsies (36.5%) showed pure UPSC histology and 39 (62%) showed mixed histological subtypes including a UPSC component. Thirty-nine of the diagnostic biopsies (63%) were described as serous, papillary, mixed serous or other histological subtype in the original pathological reports, and on review 62 of the biopsies (98.5%) contained a UPSC component. The one biopsy (1.5%) that did not contain a UPSC component was reviewed as 100% CCEC, and on hysterectomy the tumour was reviewed as 100% UPSC. The correlation between the initial biopsy and hysterectomy histology subtypes is illustrated in Figure 4.3. While there was a weak positive correlation between the diagnostic biopsies and hysterectomy diagnoses for UPSC and CCEC (Spearman's Rank Correlation Coefficient 0.47 and 0.30 respectively, $p<0.01$), the correlation is much stronger for EEC (Spearman's Rank Correlation Coefficient 0.70, $p<0.01$). In addition, those biopsies, which overestimated the amount of UPSC in the resection, were usually the same cases in which the percentage of CCEC was underestimated. In Kaplan-Meier analyses of survival, the OS and PFS were not significantly different in patients with pure and mixed histological patterns or percentage of UPSC (including less than 25% UPSC compared to more than 25% UPSC) in the diagnostic biopsies or the resections (Figure 4.4). In addition, whether UPSC was mixed with EEC or CCEC made no difference to survival. Serous endometrial intraepithelial carcinoma was identified in 2 of the hysterectomy specimens.

Figure 4.3. Percentages of Histological Subtypes in Diagnostic Biopsies Vs Resection Specimens.

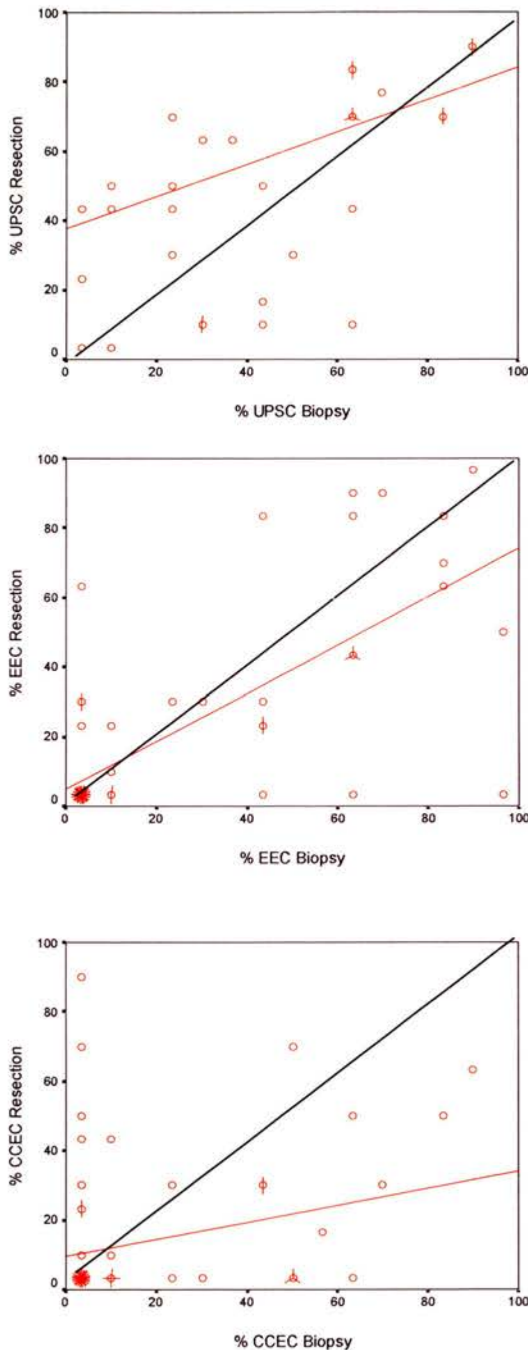
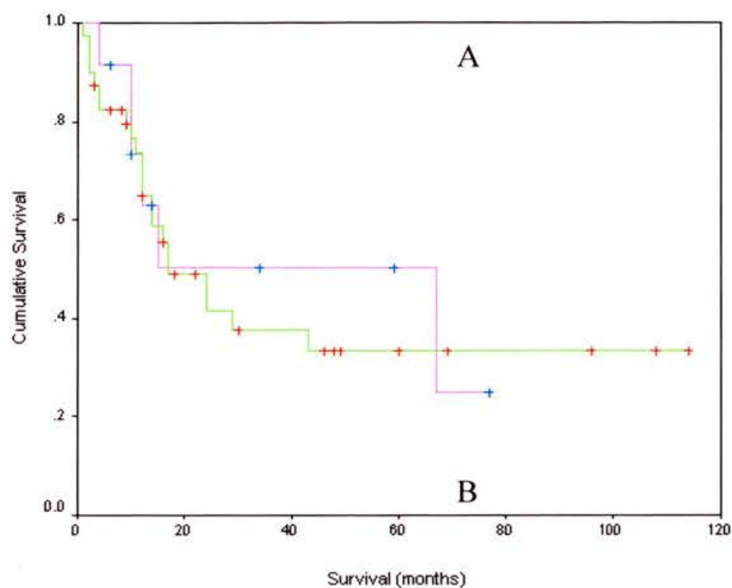
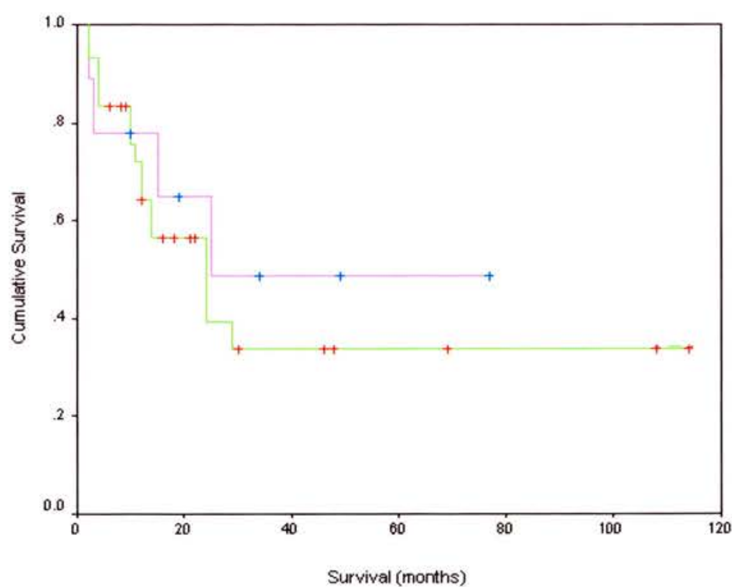


Figure 4.3. Scatterplots to represent percentages of histological subtypes in diagnostic biopsies vs resection specimens. Each spoke on a data point represents one case. Best fit lines (red) show a positive correlation between the two specimens for all three histological subtypes.

Figure 4.4. Overall Survival According to Percentage of Uterine Papillary Serous Carcinoma.





Less than 25% USPC (purple line) compared with greater than 25% USPC (green line) in pre-operative (A, p=0.76, log-rank test) and resection specimens (B, p=0.56, log-rank test).

4.3.4 Adjuvant therapy

Fifty-seven patients received adjuvant therapy. Thirty patients (52.6%) received radical radiotherapy, and 3 patients (5.2%) palliative therapy. Of the 2 patients who did not receive radiotherapy, one was deemed to have had curative surgery (Stage I disease) and the other was deemed unfit for therapy. In addition to radiotherapy 28 patients (49.1%) received adjuvant chemotherapy with different regimens including carboplatin alone, carboplatin and paclitaxel, or cisplatin (Table 4.2). Patients treated with radical radiotherapy postoperatively had a significantly better prognosis by univariate analysis ($p<0.01$, Figure 4.2). The median overall survival was 29 months in patients receiving radical radiotherapy compared with 12 months without, and the median overall survival was 67 months with palliative radiotherapy compared with 12 months without. There were no significant differences in survival in patients treated with or without chemotherapy (Figure 4.2), or between the different chemotherapy regimens.

Table 4.2. Chemotherapy Regimens by Stage.

Stage	C	C&T	Cis	Nil
I	7	2	1	13
II	4	0	1	6
III	5	3	0	5
IV	5	0	0	4

C = carboplatin, Cis = cisplatinum, C&T = carboplatin and paclitaxel.

4.3.5 Recurrence Analysis

Fifteen (22.4%) patients had recorded recurrences. Of these, 5 (33%) were distant metastases (lung, liver, omentum and neck lymph nodes) and 10 (67%) were loco-regional recurrences. Median time to recurrence was 9 months (range 4-17 months). Two omental recurrences were in patients who had had positive omental biopsies. Neither had had complete omentectomy. The recurrence rate in Stage I to IV patients was 12%, 14%, 36% and 33%, respectively. Further survival analysis was not possible due to the small numbers in each stage and non-standardised treatment regimens.

4.4 Discussion

This study of UPSC is the third largest retrospective series of its kind with focus on the effect of histopathology on outcome. The high median age of the patients, and postmenopausal status, is similar to that previously documented for UPSC {Dunton, 1991;Slomovitz, 2003}. Thirteen percent of patients had a personal history of breast cancer, identical to that observed by Slomovitz et al {Slomovitz, 2003} and similar to Barakat et al {Barakat, 1994} who observed a similar rate of low- and high-risk endometrial subtypes in breast cancer patients regardless of tamoxifen use. Following case reports and studies on Ashkenazi Jews, it was initially thought that mutations in the BRCA gene might account for this finding {Geisler, 2001;Lavie, 2000}. However, a larger study by Goshen et al {Goshen, 2000} of 56 patients with both UPSC and breast carcinoma (not limited to Ashkenazi Jews) showed that although women with mutations in the BRCA1 gene have an increased incidence in ovarian serous papillary carcinoma and endometrial cancer, there was no specific increased risk in the development of UPSC. In addition, they failed to demonstrate mutations in either BRCA1 or BRCA2 genes in these patients. They postulated that either the BRCA mutations occurring in these patients were out with the scope of the screening techniques, or that the association between UPSC and breast cancer may be due to the presence of mutations in a variety of other cancer predisposing genes such as P53 and cerbB2.

Only 63% of the biopsies were diagnosed as pure or mixed UPSC histological patterns, in line with previous reports {Bristow, 2001;Carcangiu, 1992;Goff, 1994;Sherman, 1992;Slomovitz, 2003;Tay, 1999;Kelly, 2005}, which have reported the accuracy of pre-operative biopsy from 65-93%. Most biopsies not explicitly

stated as UPSC were given Grade 3 endometrial carcinoma diagnoses or classified according to the other major histological subtype, usually CCEC. However, on retrospective review of the biopsies, only one (1.5%) of the pre-operative biopsies did not contain any UPSC, according to traditional diagnostic criteria, and the major pattern in this case was CCEC. This may be explained by the heterogeneity of the disease.

We sought to evaluate whether 25% UPSC pattern, the value above which the tumour is classified as UPSC, is prognostically valid {Walker, 1982}. We found that the survival of patients with less than 25% or more than 25% UPSC in the biopsy or the resection was the same, suggesting that tumours should be classified, and managed, as UPSC regardless of the percentage of UPSC in the pathological specimen. Our lowest percentage of UPSC used was 5%. In addition, in keeping with previous reports, pure or mixed histological patterns did not show any survival difference, and survival was the same regardless of whether UPSC was mixed with a high-risk subtype, such as CCEC, or a low-risk subtype, such as EEC {Slomovitz, 2003;Goff, 1994}, suggesting that UPSC is biologically dominant in heterogeneous tumours.

We found a low number of patients had complete surgical staging (40% omental staging, 11.9% pelvic node clearance, 50% cytology), which may in part be explained by the changing surgical practice over the period analysed, and difficulties with the interpretation of the diagnostic biopsies. Whilst this raises the possibility that the patients might not be accurately surgically staged, our survival rates are

consistent with the generally accepted long-term survival rates of 35-50% for Stage I and II UPSC and 0-15% for patients with Stage III and IV disease {Nicklin, 1996}. Gehrig et al {Gehrig, 2003} showed that the sensitivity of a visually negative omentum was 89%, concluding that, with a microscopic metastasis rate of 4%, surgical sampling does not need to be included in the routine surgical staging of UPSC. The clinical consequences of under-staging is underscored by the fact that in our data two recurrences (13%) occurred in biopsy positive omenta which were not removed by omentectomy, and these two patients had Stage III disease which, while still a poor prognosis group, may have benefited from additional abdominal surgical clearance. Omentectomy may result in better local control of disease and more directed and appropriate adjuvant therapy. Unfortunately our data were too small to establish a link between omentectomy and survival.

The rate of pelvic lymphadenectomy was low, but it is difficult to draw any conclusions from this. There has been ongoing discussion about the value of pelvic lymphadenectomy. Slomovitz et al {Slomovitz, 2003}, reported 19% lymph node involvement in patients without myometrial invasion and Huh et al {Huh, 2003} demonstrated no pelvic side wall failures following observation in a population at risk for recurrence, and found in two of the patients who had not had formal pelvic lymphadenectomy, pelvic recurrence. However, the first results of the MRC-ASTEC trial, the first (and currently, the only) randomised trial investigating the role of lymphadenectomy in clinical Stage 1 endometrial carcinoma have shown that there was no benefit of lymphadenectomy, with 3-year overall survival rates of 89% (TAH BSO alone) and 88% (TAH, BSO and lymphadenectomy). In addition the 3-year

recurrence free survival was also better in the TAH BSO alone arm and relapse rates were similar (H Kitchener, oral presentation at the European Society for Gynecologic Oncology, Istanbul, September 2005).

We did not show any significant impact of adjuvant radiotherapy or chemotherapy on PFS or OS. Ramondetta et al {Ramondetta, 2001} showed that single agent paclitaxel showed a tumour response in 77% of patients, and used in a neo-adjuvant fashion with cisplatin, Zanotti *et al* {Zanotti, 1999} saw responses in 8 out of 9 patients. In addition Kelly et al {Kelly, 2005} recently showed that platinum based chemotherapy improved disease free and overall survival of patients with Stage I UPSC and vaginal cuff radiation provided local control of disease. However, our study failed to show that treatment with these agents converted into an objective improvement in PFS or OS. This is probably due to the short average follow up time (14 months) and possibly the inadequate surgical staging, resulting in patients being understaged.

Twenty-two percent of patients in this study had further disease. This is far fewer than the percentage observed by other groups, who have documented recurrence rates of between 50-75%, using various adjuvant therapies, radiotherapy alone or with the addition of chemotherapy using platinum and/or paclitaxel alone, as used in our study {Sood, 2003;Zanotti, 1999;Mallipeddi, 1993;Frank, 1991}. Our results therefore compare favourably with similarly staged tumours at equivalent median follow-up, although similar to these studies Stage III and IV tumours remain a particular management problem due to higher rates of recurrence (36% and 33% for

Stage III and IV carcinomas compared with 12% and 14% for Stage I and II carcinomas, respectively). Establishing the exact reasons for favourable recurrence rates is hampered by limited patient numbers and inconsistent and non-randomised adjuvant therapies, but it appears that surgery produces good regional control in this group, with 67% locoregional control compared to the 87% observed by Sood et al {Sood, 2003}.

4.4.1 Summary

Despite having been described as a distinct clinical entity for over twenty years, UPSC remains a management challenge for the multidisciplinary team due to the paucity of understanding on the epidemiology, pathogenesis, natural history and optimal treatment strategies for this important and aggressive variant of endometrial adenocarcinoma. These data contribute to the growing body of literature on UPSC, and addresses diagnostic and treatment uncertainties for the pathology, surgical and oncological teams. This study raised awareness for the need of accurate and complete surgical staging at a local level. However, we have not altered our approach to the use of adjuvant chemoradiotherapy for patients with UPSC as our patient numbers were small with a limited follow up time. This study contributes to the increasing local, national and international awareness of the need to invest in randomised clinical research trials on UPSC, at a time where alternative treatment modalities may become increasingly effective.

Chapter 5

The Role of E Cadherin, P Cadherin and β Catenin in Uterine Papillary Serous Carcinoma and Endometrioid Endometrial Carcinoma.

5.1 Introduction

This chapter focuses on the first step on invasion; namely detachment of tumour cells from each other. Normal cell-cell adhesion determines the morphology of a tissue type and regulates major cellular processes including motility, growth differentiation and survival. Cadherins are a widely distributed family of cell-cell adhesion molecules which are composed of 3 parts, a single domain transmembrane portion, which, using calcium, binds to another transmembrane domain of a cadherin molecule and a cytoplasmic portion composed of 2 domains {Nose, 1988;Matsuzaki, 1990}. The cytoplasmic domain is connected to β- and γ-catenins which in turn bind to the actin cytoskeleton through α catenin {Nathke, 1994;Hinck, 1994;Jou, 1995}. The family of cadherins currently has more than 80 members including the classical cadherins, desmogleins, desmocollins and protocadherins. The most completely described cadherins are the classical types, Epithelial (E)-, Placental (P)- and Neuronal (N)- cadherin and all vertebrate cells seem to express at least one cadherin {Niessen, 2002;Takeichi, 1991}. Cadherin-catenin complexes also have roles in intercellular communication and modulation of cell function in both normal and malignant tissues.

5.1.1 E Cadherin

E cadherin is the most common cadherin in epithelial cells. It is made from 4.5 kb mRNA as a 135kDa precursor polypeptide, which is processed rapidly by proteolytic cleavage to the mature 120kDa form {Shore, 1991}. The E cadherin-catenin complex is necessary for cell-cell adhesion of epithelial cells. Failure to assemble the E cadherin-catenin complex or to properly connect to the actin cytoskeleton results in loss of cell adhesion, which may be involved in tumour spread {Van Roy, 1992;Birchmeier, 1993;Guilford, 1998;Perl, 1998}. The E cadherin gene (CDH1) is found on chromosome 16q22.1. CDH1 is thought to be a tumour suppressor gene, whose loss has been shown to cause tumour invasion and metastasis in various cancer models {Christofori, 1999}. In endometrial endometrioid carcinoma (EEC) loss of heterozygosity at 16q22 has been related to poor prognosis and CDH1 mutations and promoter hypermethylation have been found in a small percentage of cases {Kihana, 1996;Risinger, 1994;Moreno-Bueno, 2003;Saito, 2003}. In addition to abnormalities in promoters of the E cadherin gene, or the gene itself, the E cadherin-catenin complex can be disturbed by the action of TGF-β. Vogelmann et al {Vogelmann, 2005} demonstrated that TGF-β induced destabilisation of the E cadherin-catenin complex and thus dissociation of β catenin from α catenin and the actin cytoskeleton. Somatic mutations in the TGF-β signalling pathway are associated with loss of proliferative control, malignant progression, invasion and metastasis {Vogelmann, 2005}. They also demonstrated that the mechanism involved phosphorylation of β catenin by PTEN {Vogelmann, 2005}.

E cadherin is present in endometrial epithelium in proliferative phase and decreases in secretory phase {Shih, 2004}. It is possible that the cadherin-catenin complex may have a role in the maintenance of the normal endometrial architecture.

There is decreased expression of E cadherin in atypical hyperplasia compared to normal endometrium and even less in endometrial carcinoma {Shih, 2004;Moreno-Bueno, 2003}. Studies have also shown that loss of E cadherin expression increases with increasing grade of the endometrial carcinoma, such that the most poorly differentiated tumours have the least E cadherin expression {Moreno-Bueno, 2003;Shih, 2004;Dvalishvili, 2005}. Some studies have shown that E cadherin loss is more frequent in UPSC and clear cell carcinoma, compared to EEC {Holcomb, 2002;Moreno-Bueno, 2003}. In contrast, Demopoulos et al {Demopoulos, 1999} did not find any differential expression of E cadherin between EEC and UPSC. However, this study examined a relatively small number of tumours (14 cases of EEC and 10 cases of UPSC).

E cadherin negative endometrial tumours are more likely to be poorly differentiated, with local and distant dissemination of disease {Pijnenborg, 2004;Leblanc, 2001;Sakuragi, 1994;Holcomb, 2002}. Shih et al {Shih, 2004} found that nuclear β catenin expression was inversely correlated with E cadherin expression. However, Moreno-Bueno et al {Moreno-Bueno, 2003} did not find any link between reduced E cadherin expression and nuclear accumulation of β catenin.

As E cadherin has roles in cell-cell adhesion it is possible that the differential expression in E cadherin between EEC and UPSC may be, in part responsible for the poor prognosis of UPSC and increased propensity for myometrial and distant spread.

5.1.2 P Cadherin

P cadherin is expressed in trophoblast and shows restricted expression in epithelia, being expressed predominantly in basal cells of bronchial and mammary epithelium {Smythe, 1999;Deugnier, 1999}. This basal expression suggests that P cadherin may not only have a role in cell-cell adhesion, but also may have a role in differentiation and cell growth. Studies on pancreatic cell lines demonstrated that over expression of P cadherin caused increased motility of cells and accumulation of cytoplasmic p120ctn, a protein involved in cell-cell adhesion which acts by stabilising cadherin and regulating cadherin turnover {Taniuchi, 2005;Kowalczyk, 2004}.

Although P cadherin is similar to E cadherin the pattern of expression in malignancy and in particular in both normal and malignant endometrium is less well defined. An immunohistochemical study by Van Der Linden et al {Van der Linden, 1994} showed similar staining characteristics of P cadherin to E cadherin in normal endometrium and they suggested that P cadherin might play a role in maintenance of proliferative endometrial glands. P cadherin up regulation has been demonstrated in inflammatory bowel diseases and some neoplasias such as breast and cervical adenocarcinomas {Sanders, 2000;Palacios, 1995;Han, 2000}. In addition up regulation of P cadherin has been found, in increasing frequency, in atypical endometrial hyperplasia, EEC and in a group of "non EEC", which included UPSC and clear cell carcinoma {Moreno-Bueno, 2003}. Interestingly in breast and cervical adenocarcinoma and "non EEC" increased P cadherin expression is related to cell proliferation, dedifferentiation, and negative oestrogen and progesterone receptors, and in addition, increased P cadherin has been shown to confer poor prognosis in

breast cancer {Han, 2000;Gamallo, 2001}. In contrast decreased expression of P cadherin has been described in both oral squamous cell carcinoma and melanoma. In addition P cadherin loss is associated with poor prognosis in oral squamous cell carcinoma {Lo, 2005}. Recent studies have shown that a truncated form of the N terminal part of P cadherin is expressed by human melanoma cell lines and tissue from melanomas, but not by normal melanocytes or keratinocytes {Bauer, 2005;Hoek, 2004}. In addition, analysis showed that melanoma cells, rather than binding this short form of P cadherin to the cell membrane, which classically occurs in melanocytes, secreted it. The secreted form of P cadherin was shown to regulate the homophilic interaction between cadherin molecules by antagonising their homophilic binding, acting as a dominant negative form to interrupt cell-cell attachment. In addition to the shift from the membranous form to the secreted form expression of P cadherin is down regulated in malignant melanoma {Hoek, 2004}.

5.1.3 β Catenin

β catenin has roles in both cell-cell adhesion and intracellular signalling. It controls E cadherin mediated cell adhesion at the plasma membrane and mediates the interplay of adherens junction molecules with the actin cytoskeleton. In addition, β catenin acts in the Wnt signalling pathway, activating the transcription of target genes responsible for cellular proliferation and differentiation, along with LEF/TCF (lymphoid enhancer factor/T-cell factor) DNA binding proteins {Morin, 1997;Rubinfeld, 1997}. The signalling function of β catenin is mainly regulated by altering its stability and Wnt signalling induces the stabilisation of the free cytoplasmic pool of β catenin {Polakis, 2000}. In the absence of Wnt signalling, β

catenin is rapidly degraded by a destruction complex consisting of adenomatous polyposis coli (APC), axin, glycogen synthase kinase beta, conductin and casein kinase {Behrens, 1998;Kishida, 1998;Liu, 2002}.

Both functions of β catenin are deregulated in malignancy leading to the loss of both cell-cell adhesion and to the increased transcription of Wnt target genes. Mutations that activate the Wnt-β catenin pathway promote stabilisation of β catenin and induce its nuclear accumulation {Munemitsu, 1995}. This results in activated gene transcription, altered cell migration and cell polarity. Inactivating mutations in the APC gene product, a protein that promotes degradation of cytoplasmic β catenin, leads to an accumulation of cytoplasmic β catenin {Munemitsu, 1995;Morin, 1997;Rubinfeld, 1997}. However, neither LOH nor promoter hypermethylation of APC is associated with nuclear expression of β catenin {Koppert, 2004}. Other mutations that decrease the degradation of β catenin include those that affect the function of conductin or axin {Liu, 2000;Satoh, 2000;Jin, 2003}.

β catenin co-localizes with E cadherin and γ catenin at the cell membrane in all phases of the menstrual cycle {Nei, 1999;Miyamoto, 2000}. In addition, nuclear β catenin expression has been observed in the proliferative phase, suggesting a role for the Wnt signalling pathway in the normal endometrium {Miyamoto, 2000}. Chen et al {Chen, 1998} demonstrated that progesterone, but not β oestradiol increased β catenin mRNA levels in cultured human endometrial stromal cells and this finding is supported by Fujimoto et al {Fujimoto, 1998} who demonstrated that β catenin and E cadherin levels were less in the proliferative phase, where progesterone levels are

comparatively higher compared to the secretory phase, where oestrogen levels are comparatively higher.

The activation of the Wnt signalling pathway due to β catenin mutations has been implicated in the development of some endometrial carcinomas {Kariola, 2005}. Moreno-Bueno et al {Moreno-Bueno, 2004} showed that nuclear β catenin expression was found in 31% of EECs and 3% of "non EECs". However, up to 25% of endometrial carcinomas have β catenin nuclear accumulation without evidence of β catenin mutations, suggesting alterations in other molecules that can modulate the Wnt pathway such as APC, γ catenin, and axin {Moreno-Bueno, 2004}. Pijnenborg et al {Pijnenborg, 2004} found that nuclear localization of β catenin, associated with mutations in the β catenin gene and mutations in the APC gene are not predictive for recurrent disease.

In vitro studies on breast cell lines have shown that decreased E cadherin can augment β catenin oncogenic signalling {Yang, 2001} and Shih et al {Shih, 2004} demonstrated an inverse correlation between nuclear β catenin and E cadherin expression in normal and malignant endometrium. However, Moreno-Bueno et al {Moreno-Bueno, 2003} did not find any link between reduced E cadherin expression and nuclear accumulation of β catenin.

Abnormal expression of E cadherin and α-, β-, and γ-catenins has been shown to be associated with poor prognosis in many malignancies, including endometrial and ovarian cancer {Bremnes, 2002;Lim, 2002;Umbas, 1994;Zhao, 2003;Bringuier, 1993;Ropponen, 1999;Aaltomaa, 1999;Bohm, 2000;Zhou, 2002;Lee, 2002;Pantel, 1998;Shimazui, 1997;Endo, 2000;Voutilainen, 2006}.

5.1.4 Summary

Previous studies examining the expression of E cadherin, P cadherin and β catenin have concentrated on EEC and its precursors and in those papers which have also looked at UPSC, the numbers of cases have been small, or they have been incorporated into a "non EEC" group alongside other carcinomas such as clear cell carcinoma. Whilst Holcomb and Moreno-Bueno et al {Moreno-Bueno, 2003;Holcomb, 2002} showed that E cadherin expression was lower in "non EEC" compared to EEC, Demopoulos et al {Demopoulos, 1999} failed to make this distinction. Therefore this chapter aims to clarify whether or not E cadherin expression is lower in UPSC compared with EEC and also to determine if there is any differential expression of E cadherin between the central viable part of the tumour and at the invasive edge.

The expression and role of P cadherin in endometrial carcinomas is unclear, although some studies have proposed that increased expression of P cadherin may be related to increased cell proliferation, dedifferentiation and negative ER and PR, and in the case of breast cancer may be associated with poor prognosis. This chapter aims to determine whether or not P cadherin is upregulated in UPSC compared with EEC and also if there is differential expression of P cadherin between the central viable part of the tumour and at the invasive edge. As β catenin forms complexes with both E cadherin and P cadherin as part of its role in cell-cell adhesion, its expression was also studied in order to determine if there was differential expression between UPSC and EEC, whether or not there was an inverse correlation between E cadherin expression and β catenin expression and also to determine if there was any

difference in expression of β catenin between the viable central part of the tumour and at the invasive edge.

5.2 Materials and Methods

5.2.1 Tissues

Tissues were obtained as described in "Materials and Methods", Section 2.1.

5.2.2 Tissue Microarray

The tissue microarray was constructed as described in "Materials and Methods", Section 2.2.

5.2.3 Immunohistochemistry

Immunohistochemistry was performed for E cadherin, P cadherin and β catenin using the technique described in "Materials and Methods", Sections 2.3.9, 2.3.10 and 2.3.11 respectively.

5.2.4 Scoring

Scoring of antibody expression was assessed using the technique as described in "Materials and Methods", Section 2.4.

5.2.5 Statistical Analysis

Statistical analysis was performed as described in "Materials and Methods", Section 2.5.

5.3 Results

5.3.1 Intertumoural Variability

E cadherin expression in both EEC and UPSC was significantly lower than that in the normal endometrial epithelium where the score for expression was uniformly 9 (p values <0.00 for both tumour types, Independent T Test, Figure 5.4). Significantly greater E Cadherin expression was present in EEC compared to UPSC ($p = 0.03$ Independent T Test, Table 5.1) and significantly less expression of P cadherin was noted in EEC compared to UPSC ($p = 0.013$ Independent T Test, Table 5.1). There was no significant difference in β catenin expression between UPSC and EEC. Scatterplots and Pearson pairwise correlation coefficient showed a direct correlation between β catenin immunohistochemical staining and E- and P cadherin expression for both EEC and UPSC. Of the UPSC cases, correlation between β catenin and P cadherin showed a correlation coefficient of 0.685 and a p value of <0.000, and between β catenin and E cadherin showed a correlation coefficient of 0.389 and a p value of 0.001 (Pearson Pairwise Correlation Coefficient).

Of the EEC cases, correlation between β catenin and P cadherin showed a correlation coefficient of 0.705 and a p value of 0.001, β catenin and E cadherin immunohistochemistry showed a correlation coefficient of 0.767 and a p value of <0.000 (Pearson Pairwise Correlation Coefficient).

Table 5.1. Expression of E Cadherin, P Cadherin and β Catenin in the Central Part of the Tumour in UPSC and EEC.

Antibody	UPSC	EEC
E Cadherin	*7.06 SD 1.69	*7.41 SD 2.91
P Cadherin	**7.53 SD 1.9	**7.24 SD 3.1
β Catenin	7.04 SD 2.0	7.78 SD 1.7

SD = Standard Deviation

*P = 0.03 (Independent T Test)

**P = 0.013 (Independent T Test)

5.3.2 Intratumoural Variability

5.3.2.1 Uterine Papillary Serous Carcinoma

Although there was decreased E cadherin and P cadherin expression at the invasive edge compared to the viable central part of the tumour, Wilcoxon Signed Ranks Test failed to reach significance (Table 5.2). All E cadherin and P cadherin expression was membranous (Figures 5.1 and 5.2 respectively).

There was significantly greater expression of β catenin at the invasive edge compared to the viable central part of the tumour ($P = 0.03$, Wilcoxon Signed Ranks Test, Table 5.2). However, all β catenin expression was membranous (Figure 5.3).

5.3.2.2 Endometrioid Endometrial Carcinoma

Although there was decreased E cadherin and P cadherin expression at the invasive edge compared to the viable central part of the tumour, Wilcoxon Signed Ranks Test failed to reach significance (Table 5.2). All E cadherin and P cadherin expression was membranous (Figures 5.5 and 5.6 respectively). There was no difference in expression of β catenin between the viable part of the central tumour or the invasive edge. Two of the twenty EEC cases showed nuclear β catenin expression (Figure 5.7). The remainder of the EEC cases showed membranous β catenin staining.

Figure 5.1

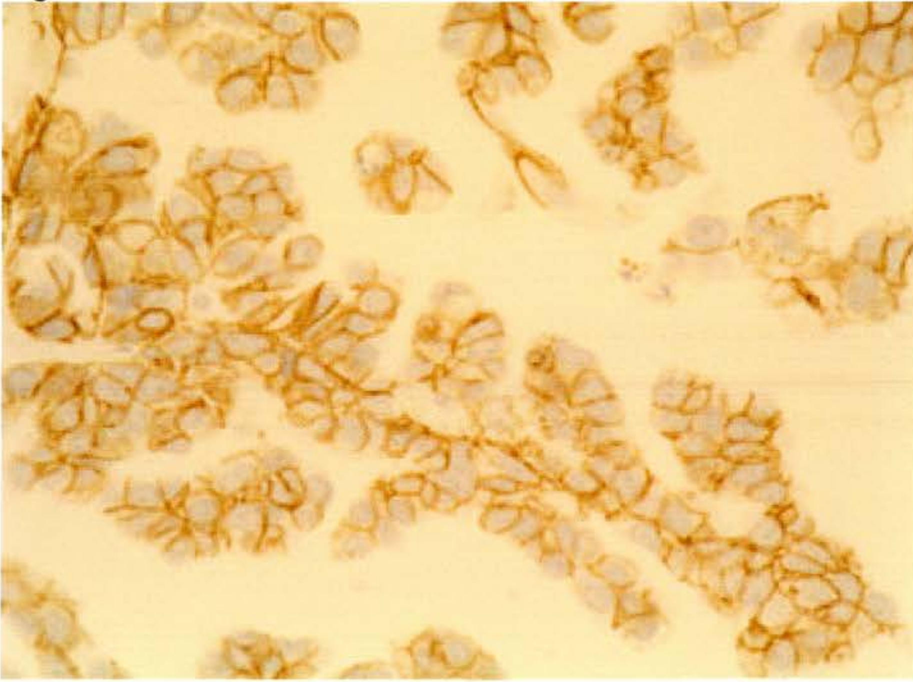


Figure 5.2

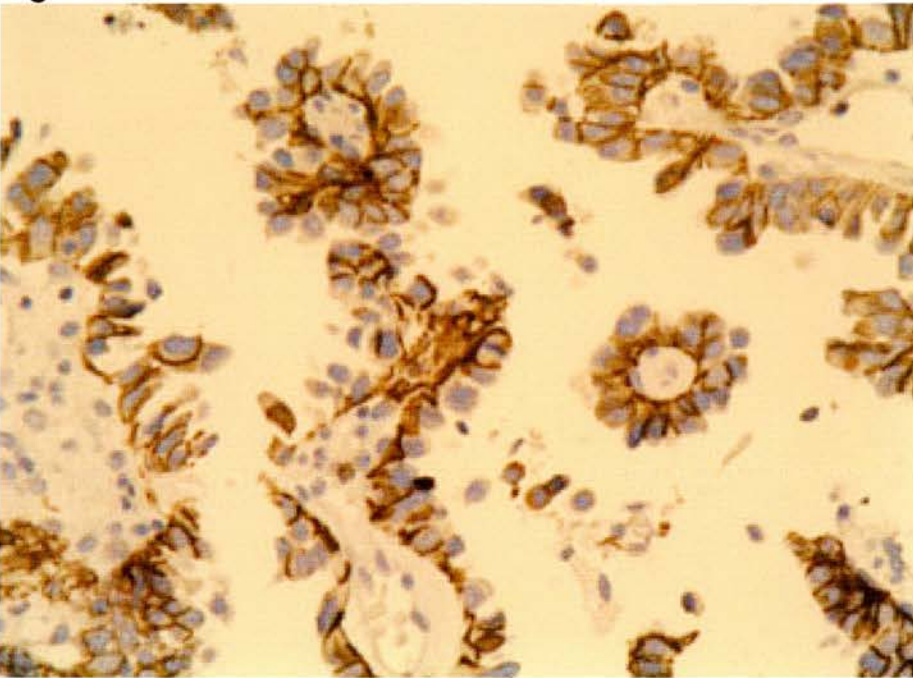


Figure 5.1. Membranous E Cadherin Expression in UPSC (Magnificationx40).

Figure 5.2. Membranous P Cadherin Expression in UPSC (Magnificationx40).

Figure 5.3

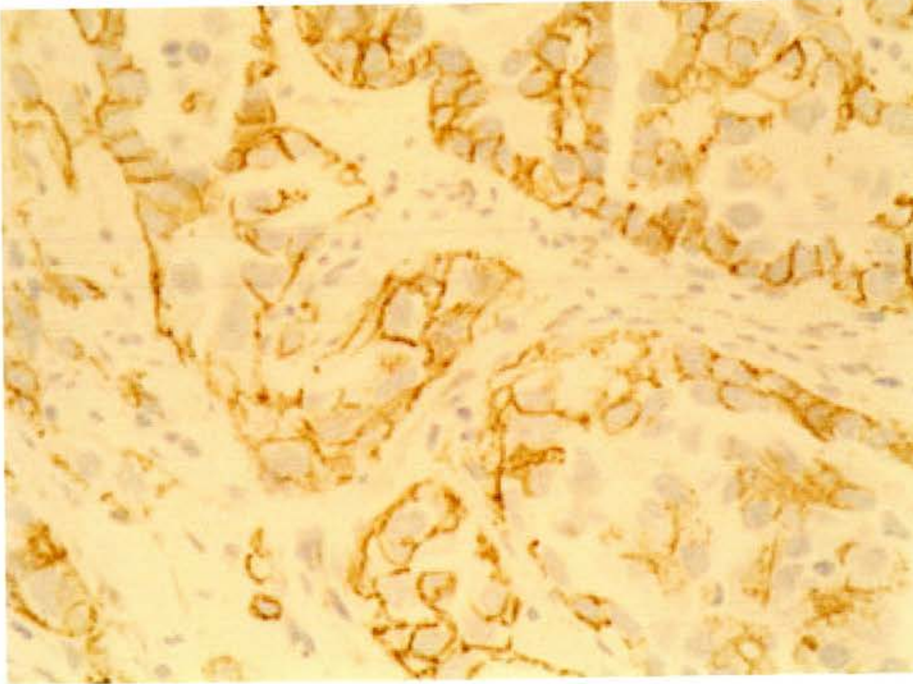


Figure 5.3. Membranous β catenin expression in UPSC (Magnificationx40).

Table 5.2. Expression of E Cadherin, P Cadherin and β Catenin in the Central Part of the Tumour and at the Invasive Edge.

Antibody	UPSC		EEC	
	Tumour	Invasive Edge	Tumour	Invasive Edge
E Cadherin	7.06 SD 1.69	6.96 SD 2.27	7.41 SD 2.91	5.46 SD 3.81
P Cadherin	7.53 SD 1.9	6.26 SD 1.99	*7.25 SD 3.1	*4.52 SD 3.32
β Catenin	**7.04 SD 2.0	**7.94 SD 1.65	7.78 SD 1.7	7.62 SD 1.85

SD = Standard Deviation

* P = 0.08 (Wilcoxon Signed Ranks Test)

** P = 0.03 (Wilcoxon Signed Ranks Test)

Figure 5.4

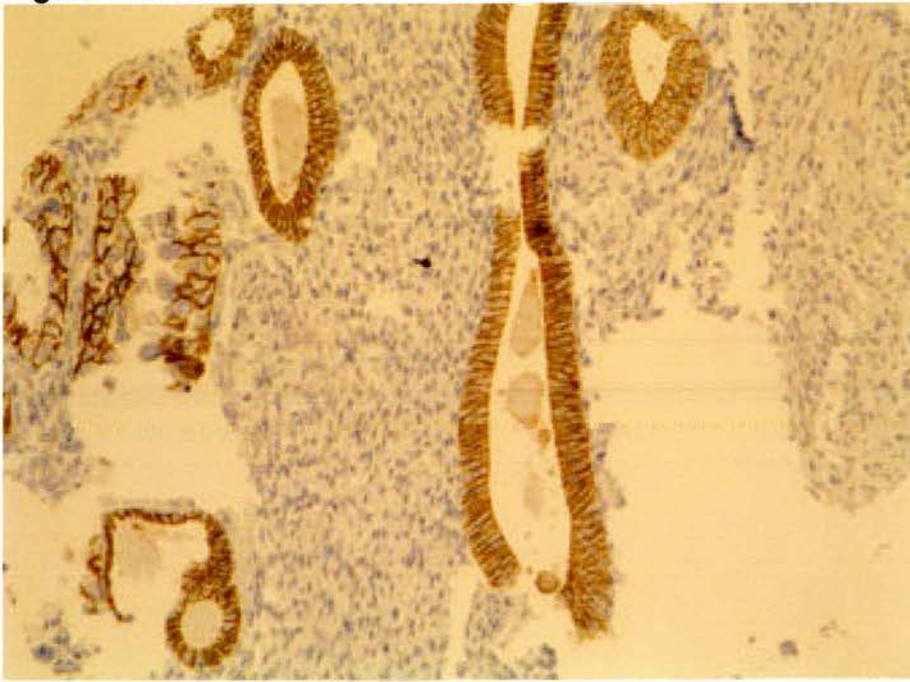


Figure 5.5

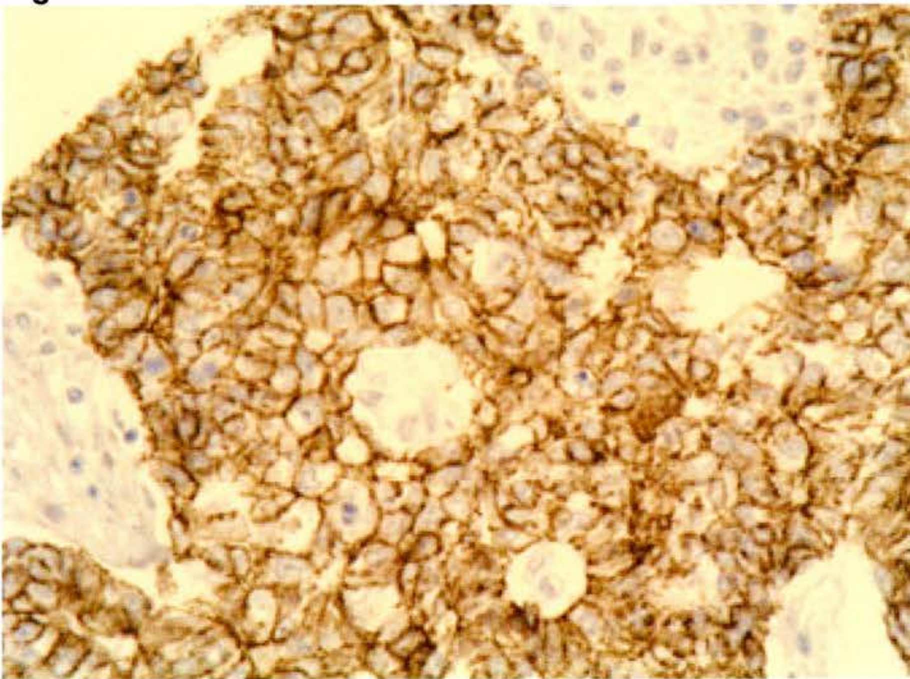


Figure 5.4. E Cadherin Expression in Normal Endometrial Epithelium (Magnificationx10).

Figure 5.5. E Cadherin Expression in EEC (Magnificationx40).

Figure 5.6

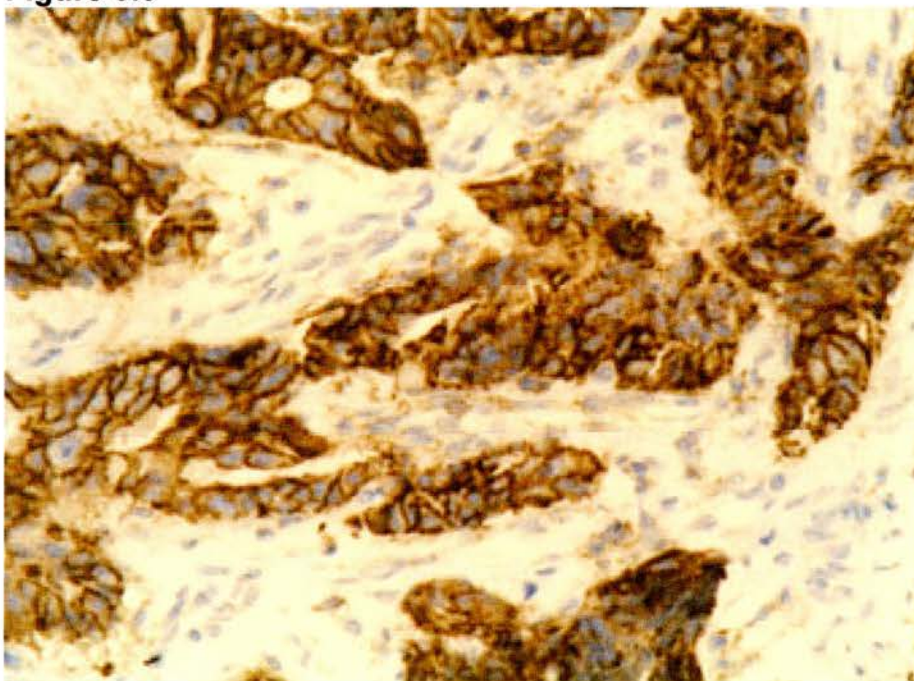


Figure 5.7

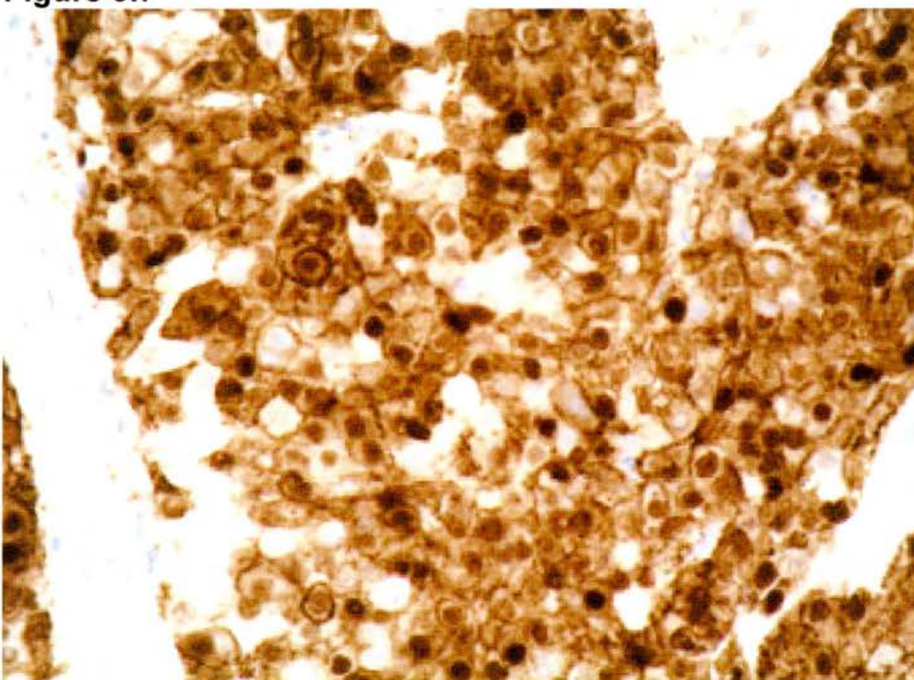


Figure 5.6. Membranous P Cadherin Expression in EEC (Magnificationx40).

Figure 5.7. Nuclear β catenin Expression in EEC (Magnificationx40).

5.4 Discussion

UPSC is an aggressive endometrial tumour with a notable propensity for lymphovascular space invasion and a poor prognosis. The cadherin-catenin complex is well described and disturbances in this are associated with a variety of cancers {Van Roy, 1992;Birchmeier, 1993;Guilford, 1998;Perl, 1998}. In addition, alteration in expression of both E- and P cadherin are associated with poor prognosis in many tumours {Pijnenborg, 2004;Leblanc, 2001;Sakuragi, 1994;Holcomb, 2002}. Along with its role in cell-cell adhesion, β catenin also affects cell proliferation via its role in the Wnt pathway. Although the expression of E-, P- cadherin and β catenin has been previously described in endometrial carcinoma, this chapter aimed to further investigate the differential expression between the tumour types and also within tumours, focussing on differences in expression between the viable central part of the tumour and the invasive edge.

We confirmed the findings of Shih et al, Holcomb et al and Moreno–Bueno et al {Shih, 2004;Holcomb, 2002;Moreno-Bueno, 2003} and demonstrated that although there was decreased E cadherin expression in both tumour types compared to the normal endometrium, the decrease in E cadherin expression was greater in UPSC than in EEC.

The finding of significantly lower E cadherin expression in UPSC compared to EEC may partly reflect the different biological properties of these tumours. EEC is a tumour which usually arises on a background of oestrogen excess, and it commonly expresses ER and PR and can produce intratumoural oestrogen using aromatase {Segawa, 2005}. On the other hand, UPSC usually arises from atrophic

endometrium, is generally ER negative and does not produce intratumoural oestrogen. We found significantly higher ER and PR expression in our EEC cases compared to UPSC (results shown in chapter 7).

To support the theory that the variation in E cadherin expression between EEC and UPSC is due to the difference hormone environments of these tumours, Fujimoto et al {Fujimoto, 1998} demonstrated that the mRNA levels of E cadherin, α- and β catenin in the endometrium in the proliferative phase were significantly less than those in the secretory phase. In addition, treatment with oestradiol dipropionate significantly reduced their levels in the secretory phase. They suggested that sex steroids could affect the adherens junction in endometrial epithelium. However a separate study showed that this effect could be reversed by treatment with progestins {Fujimoto, 1996} Therefore whilst oestrogen might play a part in lowering E cadherin in EEC, it is likely that the decrease in UPSC is due to a separate pathway.

In addition PTEN is involved in the tyrosine phosphorylation of β catenin and thus destabilisation of the cadherin catenin complex {Vogelmann, 2005}. It is well recognised that PTEN mutations occur in EEC but not in UPSC {Lax, 2004}.

Two of the EEC cases showed no E cadherin staining at all. This is an interesting finding as Pijnenborg et al {Pijnenborg, 2004} found that absence of E cadherin expression was predictive for distant metastases, but not local recurrence. However the ethics submission for this study limited the investigations to immunohistochemistry. Therefore follow up data for the patients could not be obtained.

In addition to confirming these established observations, the present study looked for differences in expression of these proteins between the viable central part of the

tumour and at the invasive edge. In both tumour types we found a decrease in E cadherin at the invasive edge of the tumour compared with the viable central part of the tumour, although the difference did not reach significance for either UPSC or EEC. The loss of E cadherin at the invasive edge of these tumours might reflect decreased cell-cell adhesion at the invasive edge and thus play a part in the mechanism of invasion for both tumours.

We demonstrated P cadherin expression in both EEC and UPSC. This is in keeping with previous studies on inflammatory bowel disease and in some cancers including breast and cervical adenocarcinoma where up regulation has been demonstrated {Sanders, 2000;Palacios, 1995;Han, 2000}. P cadherin expression was also significantly higher in UPSC compared to EEC and this is supported by previous studies, which have shown an association with negative ER and PR status. We also noted a significant decrease in P cadherin expression at the invasive edge of the EEC compared with the viable central part of the tumour. Although there was a decrease in expression between the viable central part of the tumour and at the invasive edge of the UPSC cases, this did not reach significance. The decrease in P cadherin at the invasive edge of both tumours raises the possibility that its main role in the viable central part of the tumours is related to cell proliferation and dedifferentiation. However, as studies on melanoma cells have shown secretion of a truncated form of P cadherin, which disrupts cell-cell adhesion {Bauer, 2005;Hoek, 2004}, it is possible that the decreased expression of P cadherin at the invasive edge of the tumours is due to a similar process, which cannot be detected in this study as the antibody used in this study binds with the C terminal rather than at the N terminal. It

would be interesting to further investigate this finding and to see if secretion of the truncated form also occurs in endometrial carcinoma. However, this is beyond the scope of this chapter.

β catenin was present in both UPSC and EEC. This is in keeping with the published literature {Shih, 2004;Moreno-Bueno, 2003;Moreno-Bueno, 2002;Palacios, 2001}. All UPSC cases and the majority of EEC cases demonstrated membranous expression of β catenin and only 2 EEC cases (10%) demonstrated nuclear expression. Our finding that 10% of EEC cases showed nuclear expression is less than that in the literature where nuclear β catenin expression has been demonstrated in 31% of EECs and 3% of non-endometrioid endometrial carcinomas {Nei, 1999;Moreno-Bueno, 2004;Moreno-Bueno, 2002;Palacios, 2001;Moreno-Bueno, 2003}. It is possible that this discrepancy is due to our case selection of only high grade EEC, and β catenin mutations may be more important in the development of Grade 1 and Grade 2 EEC. In addition, those cases, which showed nuclear β catenin expression, maintained this pattern of expression in both the viable central part of the tumour and at the invasive edge. There were no cases where the pattern of expression changed between the central part of the tumour and at the invasive edge, suggesting that β catenin mutation is an early event in the development of endometrial carcinoma and does not influence the capacity for invasion. Nei et al {Nei, 1999} showed that β catenin expression was highest in those cases with nuclear β catenin rather than the samples with membranous staining using western blotting. They postulated that the β catenin /Wnt-1 signal transduction was highly activated in carcinogenesis of the endometrium.

Although there was a lower level of expression of β catenin in UPSC compared to EEC, this did not reach significance. This difference may be due to the fact that EEC is associated with ER and PR excess, whereas UPSC classically is an ER and PR negative tumour. Progesterone, but not β oestradiol has been shown to increase β catenin mRNA levels in cultured human endometrial stromal cells {Chen, 1998}, and β catenin levels are known to be higher in the proliferative phase of the menstrual cycle rather than the secretory phase {Fujimoto, 1998}. Therefore it is possible that the differential secretion may be due to increased expression of PR and thus increased sensitivity to any circulating progesterone in EEC compared to UPSC.

As well as roles associated with cadherin function, β catenin is also involved in intracellular signalling, as it is a member of the Wnt pathway, which controls cell proliferation. The activation of the Wnt signalling pathway due to β catenin mutations has been implicated in the development of some endometrial carcinomas {Kariola, 2005}. However, up to 25% of endometrial carcinomas have β catenin nuclear accumulation without evidence of β catenin mutations, suggesting alterations in other molecules that can modulate the Wnt pathway such as APC, γ catenin and Axin {Koppert, 2004;Liu, 2000;Satoh, 2000;Jin, 2003}.

Some studies have shown an inverse correlation between nuclear β catenin expression and E cadherin expression in normal endometrium and endometrial cancers {Shih, 2004}. However, other studies have not demonstrated this {Moreno-Bueno, 2003}. As only 10% of our EEC cases and none of the UPSC cases showed

nuclear β catenin expression, statistical associations could not be determined. As UPSC shows a greater loss of E cadherin than EEC, but not a correspondingly higher incidence of nuclear β catenin expression, then it is possible that the correlation demonstrated by Shih et al {Shih, 2004} may only be relevant to EEC. To support this, some in vitro studies have demonstrated that cell lines with inactive E cadherin as a result of CDH1 mutations do not display constitutive Wnt signalling {van de, 2001}.

The membranous β catenin expression at the invasive edge of UPSC was significantly higher than that seen in the central viable part of the tumour. This was in contrast to the pattern of expression of E cadherin and P cadherin in UPSC where both showed a non-significant decrease in expression in at the invasive edge. This pattern of expression was similar to that seen in EEC where there was no difference in β catenin expression between the viable central part of the tumour and at the invasive edge, whereas both E cadherin and P cadherin expression was significantly lower at the invasive edge of the tumour. The decrease in E cadherin and P cadherin expression relative to β catenin raises the possibility that the relative increase in β catenin expression at the invasive edge of the tumours is due to activation of intracellular signalling along a separate pathway such as the Wnt signalling pathway.

5.4.1 Summary

This chapter has confirmed that E cadherin, P cadherin and β catenin are expressed in high grade endometrial carcinoma and that there is differential E cadherin and P cadherin expression between UPSC and EEC. This is in part may be due to the important role of oestrogen and progesterone in the development and progression of

EEC compared to UPSC. In addition we have shown alteration of expression of E cadherin, P cadherin and β catenin between the viable central part of the tumour and the invasive edge, supporting the theory of the invasive subclone acquiring mutations in order to invade and metastasise.

Chapter 6

The Expression of CD98 and Galectin-3 in Uterine Papillary Serous Carcinoma and Endometrioid Endometrial Carcinoma

6.1 Introduction

Endometrioid endometrial carcinoma (EEC) is the most commonly diagnosed type of endometrial carcinoma, comprising 80% of all cases. Uterine papillary serous carcinoma (UPSC) accounts for only 10% of cases {Burton, 1998}, but it accounts for a much smaller proportion of Stage I endometrial carcinomas {Hendrickson, 1983;Bancher-Todesca, 1998;Dembo, 1985}. UPSC is an aggressive tumour, with a propensity for lymphovascular space invasion and peritoneal metastases.

Invasion and metastasis of tumour cells requires a variety of complex interactions including cell-cell interactions, cell-matrix interactions and degradation of the extra cellular matrix (ECM). For metastasis to occur, cancer cells must detach from each other, attach to matrix components, degrade the ECM and then migrate into the blood stream which involves binding to the endothelium in target organ microvessels {Al Mehdi, 2000;Chambers, 2002}. This chapter focuses on the attachment of tumour cells to matrix components. Tumour cells adhere not only to themselves but also to the ECM and endothelial cells as part of the process of invasion and metastasis and tumour cell adhesion is mediated by specific interactions between cell surface lectins and their carbohydrate ligands presented on glycoproteins and glycolipids {Glinsky, 2003;Orr, 2000;Inohara, 1996;Kannagi, 1997;Inohara, 1995}.

6.1.2 CD98.

CD98 is a disulphide-linked 125 kDa heterodimeric membrane glycoprotein composed of a glycosylated 85 kDa heavy chain and one of at least six alternative non-glycosylated 40 kDa light chains. CD98 is encoded on chromosome 11q13 {Haynes, 1981;Hemler, 1982} and it is found on the cell surface of most normal cells and some tumour cells and has been shown to be involved in various cellular activities including cellular proliferation, cell transformation, cell fusion and cell adhesion {Yagita, 1986;Haynes, 1981;Hemler, 1982;Hara, 1999;Fenczik, 1997}. CD98 was originally identified as a cell surface antigen associated with lymphocyte activation {Haynes, 1981;Hemler, 1982}. Further experiments have shown that CD98 is quickly induced from low levels in quiescent cells to higher levels, following cellular activation. It is upregulated early in transition from G0 to G1 phase of the cell cycle and remains at elevated levels until the cell cycle is complete.

CD98 has been identified as a unique and highly specific regulator of integrin affinity {Fenczik, 2001}. It has been shown to stimulate adhesion of breast cancer cells to laminin via $\alpha3\beta1$ integrin {Chandrasekaran, 1999} and recent studies have shown CD98 to co-localise with $\beta1$ integrin on the cell membrane of keratinocytes and to promote integrin-like signalling {Lemaitre, 2005;Rintoul, 2002}. It is possible that by cross-linking CD98, CD98 acts as a facilitator in the plasma membrane, clustering $\beta1$ integrins to form high density complexes, thus subsequently leading to integrin activation and adhesion, integrin signalling and anchorage independent growth. In addition, it has been shown that $\beta1$ integrin-mediated adhesion of the small cell lung cancer cell line H345 to fibronectin and

laminin can be markedly upregulated by cross-linking CD98 {Fenczik, 1997}. Kakugawa et al {Kakugawa, 2003} also demonstrated that CD98 stimulation could activate CEA-CAM-1 mediated cell adhesion independently of integrins.

The heavy chain of CD98 is an integral membrane protein with a single membrane spanning domain, classified as a type II membrane glycoprotein {Teixeira, 1987;Quackenbush, 1987;Lumadue, 1987}. Hara et al {Hara, 2000} demonstrated that truncation of the extracellular domain of the CD98 heavy chain enhanced tumorigenicity in cell lines. Both the extracellular domain and the cytoplasmic tail of CD98 appear to be crucial for its action and CD98 that lacks either a cytoplasmic domain or the unpaired cysteine in the extracellular domain disrupts virus-induced cell fusion and does not inhibit Tac-β1 suppression of integrin affinity.

Six CD98 light chains have been identified, all of which are associated with L-type amino acid transport (LAT) activity {Mannion, 1998;Kanai, 1998;Torrents, 1998;Estevez, 1998;Mastroberardino, 1998;Pfeiffer, 1998;Pfeiffer, 1999;Tsurudome, 1999}. LAT-1 protein is widely distributed on normal cells and is involved with cellular amino acid uptake. The LAT-2 protein is predominantly expressed in renal proximal tubules and the CD98 heavy chain has been shown to control delivery of the LAT-2 protein to the membrane in a *Xenopus* oocyte model system {Kanai, 1998;Mastroberardino, 1998;Mannion, 1998;Nakamura, 1999;Pineda, 1999;Segawa, 1999;Torrents, 1998;Pfeiffer, 1999;Kanai, 2000;Sato, 1999;Fukasawa, 2000;Pineda, 1999;Rossier, 1999}. In transformed cells it is thought that L-type amino acid

transporters are upregulated to support the high level protein synthesis for continuous growth and proliferation {Christensen, 1990}.

The pattern of expression of CD98 varies between tumours. Studies have shown that the CD98 heavy chain is overexpressed in oral squamous cell carcinoma and in bladder carcinomas {Kim, 2002;Kim, 2004}. Esteban et al {Esteban, 1990} found that CD98 was present on all squamous cell carcinomas of the larynx and that a diffuse pattern of expression was associated with more extensive disease and poorer differentiation. In contrast, other studies have shown down-regulation of CD98 gene expression in cell lines made from metastatic adenoid cystic carcinoma when compared to cell lines created from adenoid cystic carcinomas that had not metastasised {Huang, 2003}. To date, no studies have been published on the pattern of expression of CD98 in endometrial carcinomas. However, previous studies examining in vivo integrin expression in normal endometrium showed that β1 integrin expression was mostly seen in endometrial stromal cells {Castelbaum, 1997}. In addition, in vitro studies showed that progesterone treatment of oestradiol-primed cells resulted in increased expression of the α1β1 collagen-laminin receptor and suppression of the αvβ3 vitronectin receptor {Castelbaum, 1997}. Lessey et al {Lessey, 1995} found alteration of integrin expression between benign and malignant endometrial epithelium, with the α5β1 integrin, most commonly seen on benign endometrial stromal cells, being found in almost 20% of cases of endometrial carcinoma. They also showed that integrin expression correlated with steroid receptor status, as well as with grade, stage and depth of invasion {Lessey, 1995}.

Thus, mounting evidence suggests that CD98 may be important in cancer and inflammation through its effects on cellular activation and integrin-mediated adhesion.

6.1.3. Galectin

The galectin family of carbohydrate binding proteins currently comprises 14 members identified by their conserved sequence elements. They all have a carbohydrate binding domain, a repetitive collagen-like sequence and a functionally distinct amino-terminal domain {Hughes, 1994;Barondes, 1994}. They appear to show some tissue and developmental specificity where galectin-1 (Gal-1) is predominantly found in tissues derived from mesoderm, galectin-3 (Gal-3) is commonly found in epithelium and macrophages, as well as cartilage, galectin-4 (Gal-4) is identified in the gastrointestinal epithelium and galectin-7 (Gal-7) is found in stratified epithelium such as the epidermis. Galectins exist as monomers but can undergo homophilic binding to form polymers through interactions of either the N terminal domain or the C terminal domain {Yang, 1998;Birdsall, 2001}. They are synthesized on cytoplasmic ribosomes and following synthesis there is selective intracellular targeting of specific galectins to subcompartments of the cytosol, to distinct subcellular organelles, and to the cell membrane and membrane bound vesicles.

6.1.4. Galectin-3

Gal-3 exists in a non-phosphorylated and a phosphorylated form. The former is found exclusively in the nucleus, while the latter is found in both the nucleus and in

the cytoplasm suggesting that phosphorylation may be important for nuclear export of the protein {Openo, 2000;Cherayil, 1989}.

Possible ligands for intracellular Gal-3 include, Bcl-2, ALIX/AIP-1, Gemin4 and CBP70. Nuclear Gal-3 is thought to play a role in pre-mRNA splicing, which in turn may be related to regulation of cell growth and proliferation {Dagher, 1995}. Gal-3 may also act to increase metastatic potential of tumour cells by promoting tumour cell adhesion {Raz, 1987;Inohara, 1995}, invasiveness {Le Marer, 1996} and inducing endothelial cell proliferation and angiogenesis {Nangia-Makker, 2000}.

A further possible role for Gal-3 is in antagonising tumour cell apoptosis via Fas ligand binding and this is thought to only occur when Gal-3 is in the phosphorylated state. The exact mechanism underlying the anti-apoptotic activity of Gal-3 is unclear, but it does share a NWGR quartet in the C terminal part with Bcl-2 and this has been shown to be critical for the anti apoptotic function Bcl-2. However it is not known whether these proteins interact in vivo, as Gal-3 expression does not alter the expression levels of Bcl-2 family members including Bcl-xL and Bax {Akahani, 1997;Matarrese, 2000;Yu, 2002}.

Gal-3 expression varies between tumour types. It is highly expressed in head and neck squamous cell carcinoma, melanoma, angiosarcoma and thyroid carcinoma {Gillenwater, 1996;Raz, 1981} In vivo expression correlates with tumour cell transformation and metastatic phenotype and it has been shown to significantly enhance tumour cell adhesion to common ECM proteins {Raz, 1990;Ochieng, 1999}, increase the incidence of lung metastases {Raz, 1990} and protect cancer cells from apoptosis {Akahani, 1997;Matarrese, 2000;Yu, 2002}. In addition, pre-

treatment of tumour cells with anti-Gal-3 antibodies reduce the incidence of metastatic lung colonies by 90% {Meromsky, 1986}.

In contrast down regulation of Gal-3 has been demonstrated in small cell lung cancer, endometrial carcinoma and breast carcinoma {Buttery, 2004;van den Brule, 1996;Castronovo, 1996} and varying results have been found in colonic carcinomas {Lotz, 1993;Bresalier, 1998}. Squamous cell carcinoma and melanoma are not chemosensitive tumours whereas small cell lung cancer and breast carcinoma are characteristically chemosensitive at presentation, but develop resistance to chemotherapy following treatment. This suggests that up-regulation of Gal-3 confers a relative resistance to chemotherapy whereas down-regulation of Gal-3 may be associated with chemosensitivity.

Gal-3 has been identified in the nucleus, cytoplasm and at the cell membrane {Openo, 2000;Cherayil, 1989}.

In addition to its intracellular roles, Gal-3 is actively secreted into the extracellular tissues where potential binding proteins include CD98, laminin and fibronectin {Dong, 1997;Sato, 1994}.

Loss of nuclear staining of Gal-3 has been demonstrated with progression of disease in colonic carcinoma and squamous cell carcinoma of the tongue {Lotz, 1993;Honjo, 2000}. However, these studies had conflicting results in that the former demonstrated an overall down-regulation of Gal-3 expression whereas the latter found an associated up-regulation of Gal-3 expression.

Van Den Brule {van den Brule, 1996} found up-regulation of Gal-1 and down-regulation of Gal-3 in EEC in comparison to normal endometrium. In addition they

found those cases with only cytoplasmic expression (i.e. no nuclear staining) of Gal-3 had increased myometrial invasion compared to those cases with both cytoplasmic and nuclear expression of Gal-3. To date, no studies on the expression of Gal-3 in any other types of endometrial carcinoma have been performed.

6.1.5. Summary

This chapter attempted to determine if differences in interaction between tumour cells and the extracellular matrix were responsible for the recognised differences in clinical behaviour between UPSC and EEC. As no studies of CD98 expression in endometrial carcinomas have been published and CD98 expression varies according to tumour type in other carcinomas, this chapter aimed to determine the pattern of expression of CD98 in EEC and UPSC. As down regulation of CD98 has been found in metastatic adenoid cystic carcinoma, this chapter also focussed on differential expression of CD98 between the central viable part of the tumour and the invasive edge. Gal-3 is a recognised ligand of CD98, so it was also included in this chapter. The expression of Gal-3 has been studied in EEC, but not in UPSC. Therefore this chapter was undertaken to determine whether there is differential expression of CD98 and Gal-3 between EEC and UPSC, which might explain the increased aggressiveness of UPSC compared to EEC.

6.2 Materials and Methods

6.2.1 Tissues

Tissues were obtained as described in "Materials and Methods", Section 2.1.

6.2.2 Tissue Microarray

The tissue microarray was constructed as described in "Materials and Methods", Section 2.2.

6.2.3 Immunohistochemistry

Immunohistochemistry was performed for CD98 and Gal-3 using the technique described in "Materials and Methods", Sections 2.3.7 and 2.3.8 respectively.

6.2.4 Scoring

Scoring of antibody expression was assessed using the technique as described in "Materials and Methods", Section 2.4.

6.2.5 Statistical Analysis

Statistical analysis was performed as described in "Materials and Methods", Section 2.5.

6.3. Results

6.3.1 Intertumoural Variability

There was significantly greater CD98 expression in EEC compared to UPSC (P = 0.009, Mann Whitney U Test, Table 6.1). All CD98 expression was membranous. Figures 6.1 and 6.2 show the pattern of expression of CD98 in EEC and UPSC respectively.

Although Gal-3 expression was higher in UPSC compared to EEC, this did not reach significance. All Gal- 3 expression was either cytoplasmic and nuclear, or cytoplasmic only. There was no difference in the pattern of expression between UPSC and EEC; Figures 6.3 and 6.4 show the pattern of expression of Gal-3 in EEC and UPSC respectively. Stromal Gal-3 staining was identified in very few cases of both UPSC and EEC. The number of cases involved was too small for statistical analysis.

Table 6.1. Expression of Galectin-3 and CD98 in the Viable Central Part of the Tumour in UPSC and EEC.

Antibody	UPSC	EEC
CD98	*4.32 SD 2.48	*6.17 SD 2.53
Galectin 3	4.75 SD 2.18	3.82 SD 2.30

SD = Standard Deviation

*P =0.009 (Mann Whitney U Test)

Figure 6.1

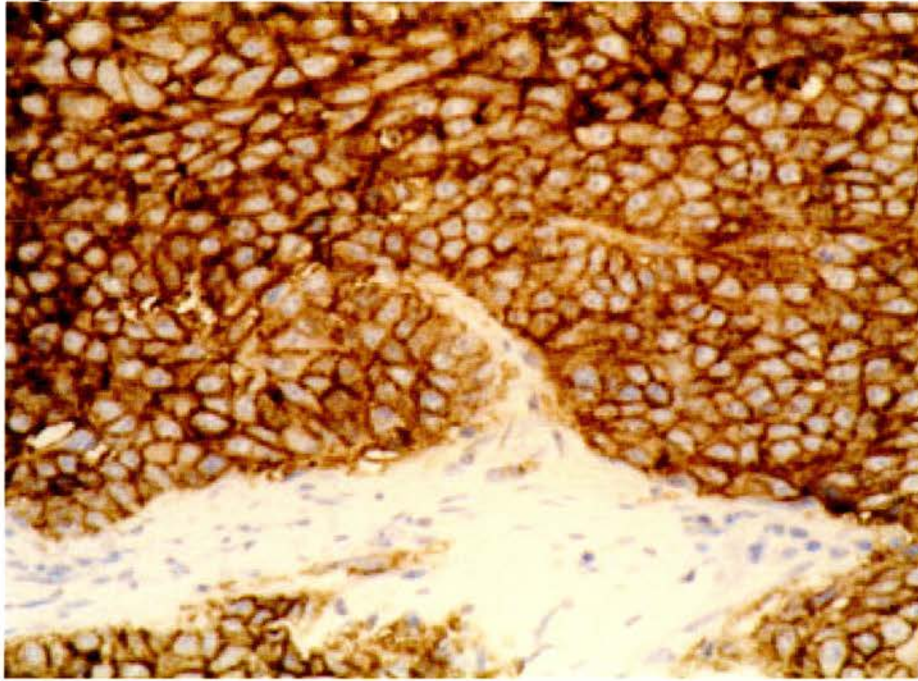


Figure 6.2

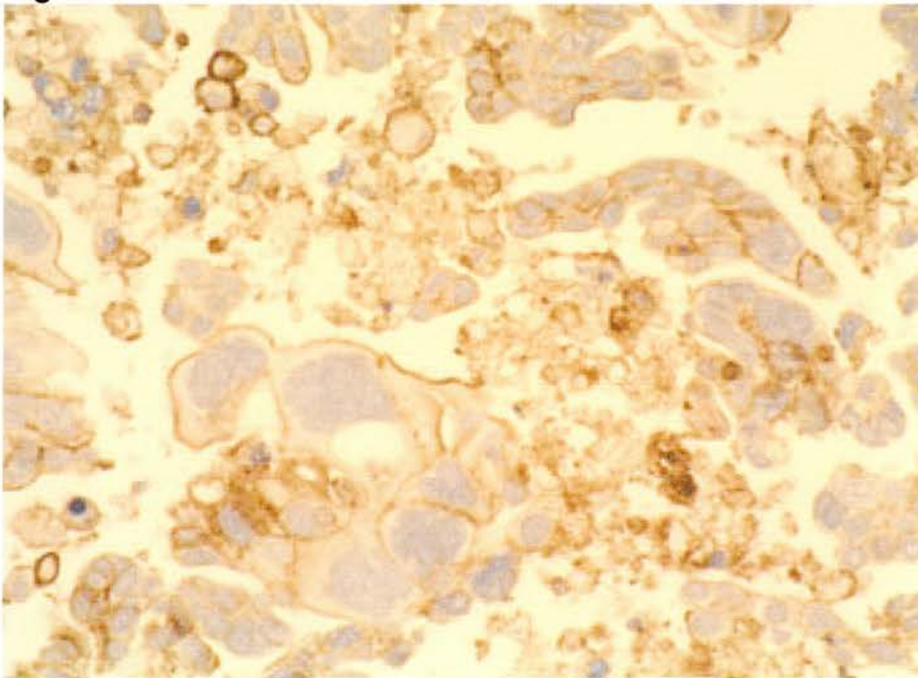


Figure 6.1. Membranous Expression of CD98 in EEC (Magnificationx40).

Figure 6.2. Membranous Expression of CD98 in UPSC (Magnificationx40).

Figure 6.3

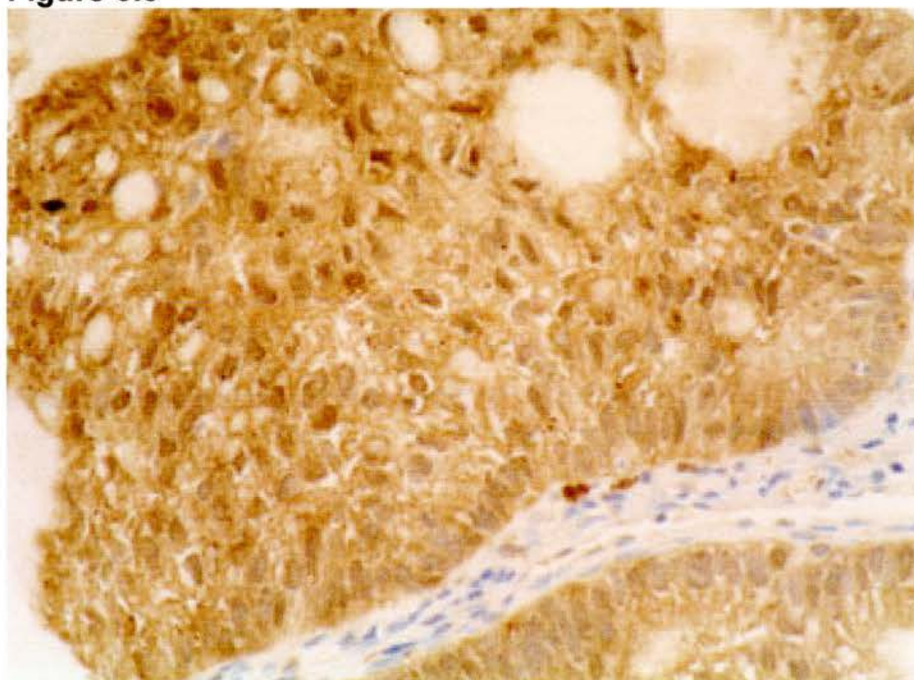


Figure 6.4

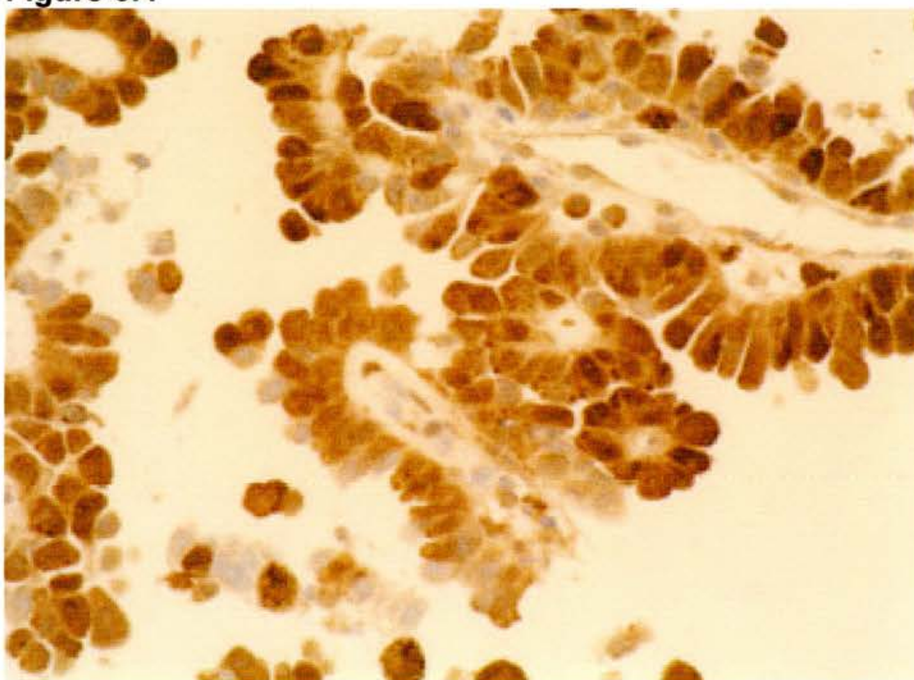


Figure 6.3. Cytoplasmic Expression of Galectin-3 in EEC (Magnificationx40).

Figure 6.4. Cytoplasmic Expression of Galectin-3 in UPSC (Magnificationx40).

6.3.2 Intratumoural Variability

6.3.2.1 Uterine Papillary Serous Carcinoma

There was significantly greater expression of CD98 at the invasive edge compared to the viable central part of the tumour ($p = 0.005$ Wilcoxon Signed Ranks Test, Table 6.2). There was significantly less Gal-3 expression in the tumour at the invasive edge compared to the viable central part of the tumour ($p = 0.031$, Wilcoxon Signed Ranks Test). Although there was a decrease in the percentage of cells showing nuclear expression of Gal-3 from the viable central part of the tumour to the invasive edge, this did not reach significance (Table 6.2).

6.3.2.2 Endometrioid Endometrial Carcinoma

No significant differences in expression of Gal-3 and CD98 were noted between the invasive edge of the tumour and the viable central part of the tumour (Table 6.2). Although there was a decrease in the percentage of cells showing nuclear expression of Gal-3 from the viable central part of the tumour to the invasive edge, from 52 % to 14%, this did not reach significance (Table 6.2).

Table 6.2. Expression of Galectin-3 and CD98 in the Central Part of the Tumour and at the Invasive Edge.

Antibody	UPSC		EEC	
	Tumour	Invasive Edge	Tumour	Invasive Edge
CD98	4.32+ SD 2.48	5.10+ SD 1.87	5.94 SD 2.53	3.92 SD 2.01
Galectin 3	4.75* SD 2.18	3.43* SD 1.68	3.77 SD 2.30	2.57 SD 1.96
Site of Gal-3 Staining (%)				
C	51	67	48	86
C&N	49	33	52	14

C= cytoplasmic staining, C&N = cytoplasmic and nuclear staining.

+P = 0.005 (Wilcoxon Signed Ranks)

*P = 0.031(Wilcoxon Signed Ranks)

6.4. Discussion

This chapter examined the immunohistochemical pattern of expression of CD98 and Gal-3 in EEC and UPSC, with the aim of determining if differential expression may, in part, contribute to the differing clinical behaviour of these tumours.

CD98 was demonstrated in all tumours examined. The finding of CD98 in both EEC and UPSC was unsurprising as CD98 plays a role at the G1/S interphase of the cell cycle in all proliferating cells.

In addition, there was significantly higher expression of CD98 in EEC compared to UPSC. The variation in expression of CD98 may be related to its association with β1 integrin. Studies have shown integrin expression to vary between benign and malignant endometrial epithelium. β1 integrin is most commonly seen on benign endometrial stromal cells, but has been found in almost 20% of cases of endometrial carcinoma {Lessey, 1995}. In addition, β1 integrin expression may be modulated by oestrogen and progesterone and Lessey et al {Lessey, 1995} found that integrin expression correlated with steroid receptor status, as well as with grade, stage and depth of invasion. Therefore if high oestrogen levels affect β1 integrin, it is possible that CD98 expression may also be upregulated by high intratumoural oestrogen.

EEC usually arises on a background of oestrogen excess, commonly expresses oestrogen receptors (ER), and can produce intratumoural oestrogen using aromatase. In contrast, UPSC usually arises from atrophic endometrium, is generally ER negative and does not produce intratumoural oestrogen. We found significantly higher ER and progesterone receptors (PR) positivity in our EEC cases compared to UPSC (results shown in chapter 7).

Alternatively CD98 expression may be due to activity of the LAT amino acid transporter system, making amino acids available for protein synthesis by proliferating tumour cells.

Significantly lower levels of CD98 were seen in the viable central part of UPSC when compared to the invasive edge, suggesting a different role of CD98 in UPSC compared to EEC. The increased expression of CD98 at the invasive edge of UPSC compared to the viable central part may reflect activation of the β1 integrin signalling pathway, increased anchorage independent growth and thus invasion. It is well known that integrin is a key factor for cell invasion and migration, allowing the cancer cell to penetrate adjacent tissues and attach itself to the target tissue's basal matrix by binding to components of the extracellular matrix {Hood, 2002}.

In contrast, our finding of increased expression of CD98 in the viable central part of EEC and a non significant decrease at the invasive edge has similarities to the findings of Huang et al {Huang, 2003} who compared expression of CD98 in both primary and metastatic adenoid cystic carcinoma. It is possible that the decrease in CD98 at the invasive edge of EEC compared to the central viable part and the metastatic adenoid cystic carcinoma compared to the primary tumour reflects a similar mechanism. The loss of CD98 might cause or enable loss of tumour cell-cell adhesion in a mechanism different to that where increased expression stimulates β1 integrin signalling and thus anchorage independent growth. The high CD98 in the viable central part of EEC may be due in part to the relative increase in PR and ER, affecting β1 integrin status and activity and therefore CD98 indirectly, but it is also

possible that CD98 plays a role in increasing nutrient uptake in proliferating cells via the LAT system.

Similarly to CD98, we identified Gal-3 expression in all cases examined. Although there was higher Gal-3 expression in UPSC compared to EEC, this difference did not reach significance. Gal-3 expression was significantly less at the invasive edge of UPSC and there was also a non-significant decrease in Gal-3 expression at the invasive edge of EEC when compared to viable central part of the tumour. This decrease in Gal-3 expression was also mirrored by the loss of nuclear expression of Gal-3 in a high proportion of both tumours at the invasive edge. Gal-3 expression was both nuclear and cytoplasmic and cytoplasmic alone. In the central part of both tumours the site of Gal-3 expression was equally split between nuclear and cytoplasmic and cytoplasmic alone, raising the possibility that Gal-3 was acting in a variety of roles including cell growth, proliferation and cell adhesion. At the invasive edge of both tumours a higher proportion of cases showed cytoplasmic expression only. However, this did not achieve significance. This non-significant loss of nuclear staining at the invasive edge is in keeping with previous studies showing an association with loss of nuclear expression and progression of disease {Lotz, 1993;Castronovo, 1996;Bresalier, 1998}. The authors of these studies suggested that nuclear expression of Gal-3 conferred specific antiapoptotic properties to the cell, whereas cytoplasmic expression conferred invasive properties to the cell.

In addition we found a significant decrease of Gal-3 expression at the invasive edge of UPSC and a non-significant decrease in Gal-3 expression at the invasive edge of EEC. This is similar to the findings of Lotz et al {Lotz, 1993} who showed an

overall down regulation of Gal-3 in colonic carcinoma along with loss of nuclear expression. Lotz et al {Lotz, 1993} suggested that nuclear localisation of Gal-3 was associated with cessation of cell division and differentiation and therefore loss of nuclear expression may be associated with increasing cell division and dedifferentiation. It would be interesting; in a further study to determine if the down regulation and loss of nuclear expression of Gal-3 in colonic adenoma-carcinoma sequence is mirrored in either the progression of endometrial carcinoma in situ (ECIS) to UPSC or in the atypical hyperplasia-carcinoma sequence of EEC.

Stromal Gal-3 was identified in very few cases of EEC and UPSC. Therefore it was not possible to determine any association between Gal-3 expression and CD98 expression and in particular to determine if Gal-3 was acting as a ligand for CD98 in these tumours.

6.4.1 Summary

This immunohistochemical study has confirmed that CD98 and Gal-3 are present in both EEC and UPSC. We found significantly higher expression of CD98 in EEC compared to UPSC. This may be due to the known relationship of CD98 with β1 integrin and indirectly of β1 integrin with ER status. Despite the lower expression of CD98 in UPSC compared to EEC we did show increased expression of CD98 at the invasive edge of UPSC. This is may be due to increased requirement of invading UPSC cells for nutrients, alternately this may reflect cross linking of β1 integrin and thus activation of β1 integrin signalling and thus anchorage independent growth. There was no significant difference in Gal-3 expression between the tumour types, but Gal-3 expression did significantly decrease at the invasive edge of UPSC

compared to the central viable part of the tumour. This decrease in Gal-3 expression also occurred to a non-significant level in EEC and was mirrored by a similar loss of nuclear expression of Gal-3. These findings raise the possibility that loss of nuclear expression of Gal-3 is associated with the development of invasive properties of these tumours.

Chapter 7

The Role of Matrix Metalloproteases -2, -7 and -9 in Uterine Papillary Serous Carcinoma and Endometrioid Endometrial Carcinoma

7.1 Introduction

Endometrial carcinoma is the most common female genital malignancy in the western world {Creasman, 2003}. There are 2 main types of endometrial carcinomas: endometrioid endometrial carcinoma (EEC) (Type I) and non-endometrioid endometrial carcinomas (Type II) which mainly consist of uterine papillary serous carcinoma (UPSC), clear cell carcinoma and malignant mixed mesodermal tumour (carcinosarcoma). EEC accounts for approximately 80% of all endometrial carcinomas and usually arises from atypical hyperplasia of the endometrium {Burton, 1998;Santin, 2002;Santin, 2003}. It is associated with oestrogen excess. UPSC accounts for approximately 10% of cases of endometrial carcinoma {Burton, 1998} and makes up the majority of the Type II carcinomas. It is an aggressive tumour, usually occurring in elderly women and is thought to arise from endometrial intraepithelial carcinoma {Wheeler, 2000;Ambros, 1995;Spiegel, 1995}. It is not associated with either atypical hyperplasia or oestrogen status. Although UPSC comprises 10% of cases of endometrial carcinoma {Burton, 1998} it accounts for a much smaller proportion of Stage I endometrial carcinomas {Hendrickson, 1983;Bancher-Todesca, 1998;Dembo, 1985}. It is upstaged at the time of surgery in 60% of cases {Gehrig, 2001;Jeffrey, 1986;Kato, 1995;Goff, 1994;Dunton, 1991}. Hui et al {Hui, 2005} examined a series of 40 cases of early uterine serous carcinoma. Nine of these were endometrial intraepithelial carcinoma

and 31 were cases of Stage Ia UPSC. They demonstrated that only when the carcinoma was confined to an endometrial polyp, did the patients have good prognosis. It is possible that this aggressive behaviour could be explained by UPSC interacting with the extracellular matrix (ECM) in a different way to EEC.

Liotta et al {Liotta, 1986} suggested a hypothetical model in which tumour invasion and metastasis resulted from repetition of three steps; adhesion of cancer cells to BM glycoproteins; degradation of BMs by specific proteolytic enzymes; and migration of cancer cells. This chapter focuses on degradation of the ECM.

The theory of tumour progression and heterogeneity is well established. Tumours are known to lose clonality early in development and generate subclones with varying characteristics from mutations. In theory only the selected subclone will possess the correct genetic characteristics in order to invade and metastasise.

7.1.2 Matrix Metalloproteases

Matrix metalloproteinases (MMPs), a family of zinc-dependent endoproteinases, are widely accepted to play a role in the invasion and metastasis of tumours {Chang, 2001;Vihinen, 2002}. Under normal physiological conditions MMPs are expressed at a very low level in adult tissues, except in tissues that undergo remodelling such as cycling endometrium, normal breast tissue at the time of involution and the skin during wound healing {Parks, 1999;Chang, 2001;Rodgers, 1993;Rodgers, 1994}. Alteration of MMP expression has been implicated in a number of diseases such as rheumatoid arthritis, osteoarthritis, chronic wounds and cancer {Chang, 2001;Vihinen, 2002;Stamenkovic, 2000;Parks, 1999;John, 2001;Jiang, 2002}. Increased MMP expression and proteolytic degradation of ECM have been detected

in a wide range of cancers, including those arising from the breast, colon, ovary and lung and MMP expression has been correlated with primary tumour growth and angiogenesis and also tumour invasion and metastasis {Chang, 2001;Vihinen, 2002;Stamenkovic, 2000;John, 2001;Jiang, 2002;Kugler, 1999;Stetler-Stevenson, 1999}.

Based on their structure and substrate specificity MMPs are divided into several groups that include collagenases (MMP-1, MMP-3 and MMP-8), gelatinases (MMP-2 and MMP-9), stromeolysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP-26), metalloelastase (MMP-12) and membrane type matrix metalloproteinases (MT-MMPs) {Chang, 2001;Vihinen, 2002;Stamenkovic, 2000;Parks, 1999;John, 2001;Jiang, 2002}.

In the past it was generally accepted that cancer cells were responsible for producing MMPs in human tumours. However, this concept underwent revision in 1990 when Basset et al {Basset, 1990} reported that the stromal fibroblasts within tumours, rather than the tumour cells themselves, were responsible for the production of MMP-11 in human breast cancer. Since then MMPs-1, -2, -3, -9, and MT1-MMP mRNA has been shown by in situ hybridization to be primarily in fibroblasts especially in proximity to invading cancer cells in a variety of tumours including breast, colon, and ovary {Polette, 1996;Nelson, 2000}. In contrast, MMP-2 mRNA has been shown in prostatic cells rather than in fibroblasts {Still, 2000}. In addition, immunohistochemistry (IHC) on a variety of human cancers has consistently demonstrated the localization of MMP protein in cancer cells. These data suggested that MMPs produced and secreted by stromal fibroblasts and inflammatory cells may bind to the cell membrane of cancer cells, thus giving them the proteases required for

invasion. To support this, Yu et al {Yu, 2000} demonstrated that MMP-2 binds to the TIMP-2:MT-MMP complex on the cell surface and that MMP-9 binds to CD44. Olson et al {Olson, 1998} also showed that MMP-9 binds to type IV collagen on the cell surface, and Barmina et al {Barmina, 1999} demonstrated that MMP-13 binds to a receptor on the cell surface prior to endocytosis and lysosomal degradation. A complicating factor is that MMP expression appears to vary among host organ microenvironments and stromal MMPs may promote metastasis to one organ in preference to another. Therefore anti-metastatic effects based on MMP inhibition may be dependent on MMPs derived from specific organ microenvironments as well as tumour cells {Shiraga, 2002}.

Although some MMPs are synthesised and secreted by cancer cells others are synthesised and secreted by stromal cells and then bind to the cell membrane of tumour cells {Basset, 1990;Polette, 1996;Nelson, 2000;Still, 2000;Yu, 2000;Olson, 1998;Barmina, 1999}. MMP expression appears to vary among host organ microenvironments and stromal MMPs may promote metastasis to one organ in preference to another. Therefore anti-metastatic effects based on MMP inhibition may be dependent on MMPs derived from specific organ microenvironments as well as tumour cells {Shiraga, 2002}.

In female reproductive tract tissues, several MMPs and tissue inhibitory metalloproteases (TIMPs) are expressed, and there is an association with events such as menstruation, abnormal uterine bleeding, ovulation, embryo implantation, cervical ripening and parturition {Martelli, 1993;Rodgers, 1994;Matrisian, 1992}.

7.1.3 Matrix Metalloprotease 2 (MMP-2)

ProMMP-2, the precursor to active MMP-2, is widely expressed in normal tissue and the MMP-2 gene has been described as a 'house-keeping' gene for its role in normal cellular processes {Matrisian, 1994}. In contrast, active MMP-2 is increased significantly in some neoplastic tissues and absent in most normal tissues. MMP-2 contributes to cell migration by a mechanism involving interaction with collagen {Xu, 2005}. MMP-2 has been demonstrated in endometrial carcinoma cells {Misugi, 2005}.

Park et al {Park, 2001} demonstrated that synthesis and secretion of stromal MMP-2 was up regulated by β oestradiol. In addition they showed that cells from an endometrial adenocarcinoma cell line invaded by recruiting MMP-2 secreted by endometrial stromal cells to their cell membrane and again, this was enhanced by the presence of β oestradiol.

MMP-2 expression is used to help differentiate high grade from low-grade endometrial carcinoma {Iurlaro, 1999;Liokumovich, 1999;Inoue, 1997}.

7.1.4 Matrix Metalloprotease 7 (MMP-7)

Altered expression of MMP-7 has been demonstrated in EEC and increased expression of MMP-7 is associated with lymph node metastasis {Yabushita, 2000;Ueno, 1999;Moser, 1999;Misugi, 2005}. In colonic adenocarcinoma MMP-7 expression is known to be increased in tumour cells but not stromal cells {Matrisian, 1994} and is associated with the risk of distant metastases and lymph node metastases {Adachi, 1999;Newell, 1994}. Matrisian et al {Matrisian, 1994} showed that induction of MMP-7 expression in tumour cells increased tumorigenicity and

metastases, while inhibition of MMP-7 led to decreased metastasis {Witty, 1994;Hasegawa, 1998}. In addition, they found that MMP-7 expression appeared to be associated with a less aggressive phenotype, despite being involved in invasion. In contrast, Misugi et al {Misugi, 2005} demonstrated that increased expression of MMP-7 was found in high grade endometrial carcinomas and this expression was also associated with tumour invasion and metastasis. Mylona et al {Mylona, 2005} detected MMP-7 expression in both tumour and stromal cells of breast carcinoma and showed a direct correlation of expression of MMP-7 with MMP-2 in the same tumour types.

Crawford et al {Crawford, 1999} described a positive correlation between nuclear β catenin protein levels and MMP-7 transcripts in colonic carcinoma. They did not find any MMP-7 expression in cells that lacked β catenin protein accumulation. In contrast, Mylona et al {Mylona, 2005} demonstrated an inverse correlation with nuclear β catenin expression in breast carcinoma.

7.1.5 Matrix Metalloprotease 9 (MMP-9)

MMP-9 expression has been shown to be altered in EEC and increased expression is associated with myometrial invasion {Yabushita, 2000;Ueno, 1999;Moser, 1999;Misugi, 2005}. In addition, Park et al {Park, 2001} showed that synthesis and secretion of MMP-9 was down regulated by β oestradiol. Cioppi et al {Cioppi, 2004} found that MMP-9 expression was higher in oestrogen receptor (ER) positive endometrial carcinomas. In addition MMP-9 has been shown to be useful in differentiating normal from sarcomatous endometrial stroma {Iurlaro, 1999;Liokumovich, 1999;Inoue, 1997}.

7.1.6 Matrix Metalloprotease 26 (MMP-26)

MMP-26 and TIMP-4 expression is elevated in endometrial carcinomas with the highest expression correlating with deeper myometrial invasion and high grade tumours. There is no difference in expression between high-grade EEC and USPC {Tunuguntla, 2003}.

7.1.7 Summary.

Although UPSC comprises only 10% of endometrial carcinomas it accounts for a greater proportion of deaths. This chapter investigated the expression of MMPs-2, -7 and -9 in UPSC and EEC, and in particular to see if there was any difference in expression between the two tumour types which might explain the observed difference in aggressive behaviour. Previous studies have shown that the expression of MMPs -2 and -7 may be upregulated by β oestradiol; therefore, we also examined the expression of ER and progesterone receptors (PR) in these tumours. β catenin expression was studied in more detail in Chapter 5, but as studies on colon and breast carcinoma have shown both positive and negative correlations between the expression of β catenin and MMP-7, any correlation between β catenin expression and any of the MMPs was noted. It is well recognised that tumours are heterogeneous and examining the central part of the tumour alone may give misleading results as regards the invasiveness of the tumour as a whole. We used tissue microarrays (TMAs), to examine a relatively large number of cases, in order to be able to demonstrate differences in expression of these proteins between the viable central part of the tumour and the invasive edge in UPSC and EEC.

7.2 Materials and Methods

7.2.1 Tissues

Tissues were obtained as described in "Materials and Methods", Section 2.1.

7.2.2 Tissue Microarray

The tissue microarray was constructed as described in "Materials and Methods", Section 2.2.

7.2.3 Immunohistochemistry

Immunohistochemistry was performed for MMPs -2, -7 and -9 and for ER and PR using the technique described in "Materials and Methods", Sections 2.3.3, 2.3.4, 2.3.5, 2.3.2 and 2.3.6 respectively.

7.2.4 Scoring

Scoring of antibody expression was assessed using the technique as described in "Materials and Methods", Section 2.4.

7.2.5 Statistical Analysis

Statistical analysis was performed as described in "Materials and Methods", Section 2.5.

7.3 Results

7.3.1 Intertumoural Variability

Significantly greater MMP-2 expression was present in EEC carcinoma cells compared to UPSC carcinoma cells ($p<0.05$ Mann Whitney U Test, Table 7.1). There was no significant difference in MMP-2 expression between UPSC and EEC stromal cells. In addition, there was no significant difference in either carcinoma cell or stromal cell expression of MMP-7 between UPSC and EEC. Although there was no significant difference in MMP-9 expression between UPSC and EEC carcinoma cells, there was significantly greater MMP-9 expression seen in EEC stromal cells ($P = 0.001$, Mann Whitney U Test, Table 7.1). There was significantly greater expression of ER and PR in EEC compared to UPSC ($P = 0.033$ and $P = 0.02$ respectively Mann Whitney U Test, Table 7.1). Scatterplots and Pearson pairwise correlation coefficient did not show any direct or inverse correlation between β catenin expression and MMPs-2, -7 or -9, in either UPSC or EEC.

Table 7.1. Expression of MMPs -2, -7 and -9 in the Viable Central Part of the Tumour in UPSC and EEC.

Antibody	UPSC		EEC	
	Tumour cells	Stromal cells	Tumour cells	Stromal cells
MMP-2	0.53* SD 0.70	1.68 SD O.67	1.43* SD 0.97	1.36 SD 0.38
MMP-7	1.81 SD 0.55	1.28 SD 0.56	1.77 SD 0.48	1.51 SD 0.59
MMP-9	0.90 SD 0.73	1.24^{+} SD 0.73	1.17 SD 0.89	2.44^{+} SD 4.05
ER	**4.38 SD 2.46		**5.85 SD 3.00	
PR	***0.96 SD 1.70		***4.67 SD 3.42	

*P < 0.05 (Mann Whitney U Test)

$^{+}$P = 0.001 (Mann Whitney U Test)

**P = 0,033 (Mann Whitney U Test)

***P = 0.02 (Mann Whitney U Test)

7.3.2 Intratumoural Variability

7.3.2.1 Uterine Papillary Serous Carcinoma

There was significantly greater MMP-2 expression in carcinoma cells at the invasive edge compared to those in the viable central part of the tumour ($p = 0.045$, Wilcoxon Signed Ranks Test). However, there was no significant difference in expression of MMP-2 in stromal cells between the invasive edge and the viable central part of the tumour (Table 7.2). MMP-2 staining was present in both the cytoplasm and at the cell membrane (Figure 7.1).

There was no significant difference in either MMP-7 or MMP-9 expression between the viable central part of the tumour or the invasive edge in either carcinoma or stromal cells (Table 7.2). Staining of both MMP-7 and MMP-9 in both carcinoma and stromal cells was cytoplasmic and membranous (Figures 7.2 and 7.3 respectively).

There was significantly greater expression of MMP-2 in stromal cells compared to carcinoma cells in both the viable central part of the tumour and at the invasive edge ($p<0.05$ and $p = 0.001$ respectively, Wilcoxon Signed Ranks Test, Table 7.3). In contrast there was significantly less expression of MMP-7 in the stromal cells compared to the carcinoma cells in both the viable central part of the tumour and at the invasive edge ($p<0.05$ and $p = 0.001$ respectively, Wilcoxon Signed Ranks Test, Table 7.3). There was significantly less MMP-9 expression in carcinoma cells compared to stromal cells in the viable central part of the tumour ($p = 0.02$, Wilcoxon Signed Ranks Test, Table 7.3), but there was no significant difference in MMP-9 expression between these cells at the invasive edge.

Table 7.2. Expression of MMPs -2, -7 and -9 in the Viable Central Part of the Tumour and at the Invasive Edge.

Antibody	UPSC		EEC	
	Tumour	Invasive Edge	Tumour	Invasive Edge
MMP-2	0.53* SD 0.70	0.79* SD 0.46	1.43 SD 0.97	1.69 SD 0.58
MMP-2 Stroma	1.68 SD 0.68	1.54 SD 0.73	1.42 SD 0.52	1.46 SD 0.94
MMP-7	1.81 SD 0.55	1.69 SD 0.85	1.77 SD 0.48	1.61 SD 0.57
MMP-7 Stroma	1.27 SD 0.83	1.21 SD 0.75	1.52 SD 0.43	1.51 SD 0.56
MMP-9	0.90 SD 0.73	1.08 SD 0.84	1.14 SD 0.89	1.09 SD 0.96
MMP-9 Stroma	1.24 SD 0.99	1.22 SD 0.76	2.51 SD 1.08	1.76 SD 0.72

SD Standard Deviation

* $P = 0.045$ (Wilcoxon Signed Ranks Test)

Table 7.3. Variation in Matrix Metalloproteinase Expression between Tumour and Stromal cells in UPSC.

Antibody	Main Tumour		Invasive Edge	
	Tumour cells	Stromal cells	Tumour cells	Stromal cells
MMP-2	0.53* SD 0.70	1.68* SD 0.68	0.79^{+} SD 0.46	1.54^{+} SD 0.73
MMP-7	1.81** SD 0.55	1.27** SD 0.83	1.69^{++} SD 0.85	1.21^{++} SD 0.75
MMP-9	0.90*** SD 0.73	1.24*** SD 0.99	1.08 SD 0.84	1.22 SD 0.76

SD Standard Deviation

*P <0.05 (Wilcoxon Signed Ranks Test)

**P <0.05 (Wilcoxon Signed Ranks Test)

***P = 0.02 (Wilcoxon Signed Ranks Test)

$^{+}$P = 0.001 (Wilcoxon Signed Ranks Test)

$^{++}$P = 0.001 (Wilcoxon Signed Ranks Test)

Figure 7.1

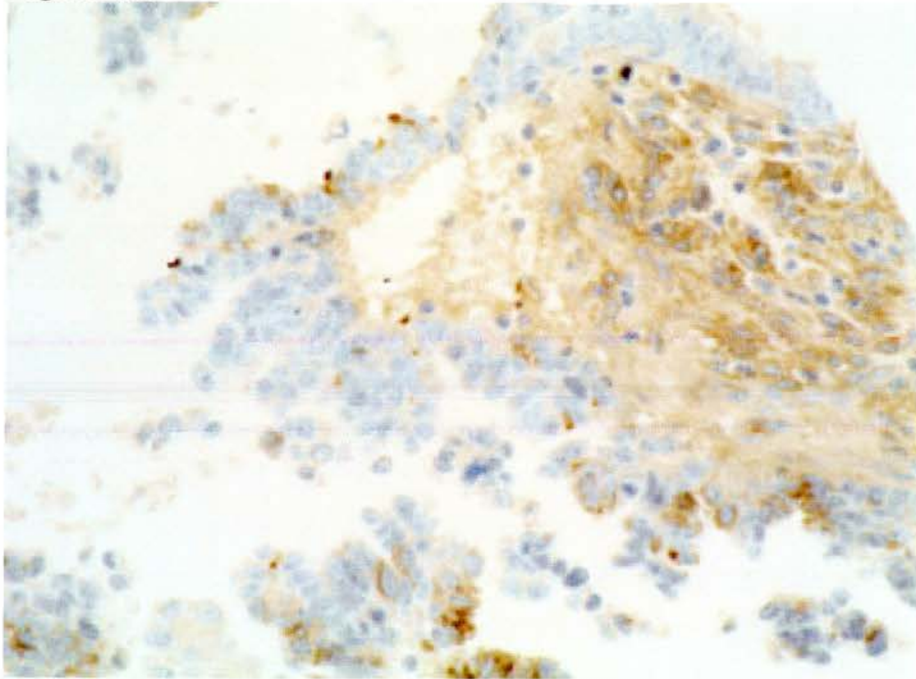


Figure 7.2

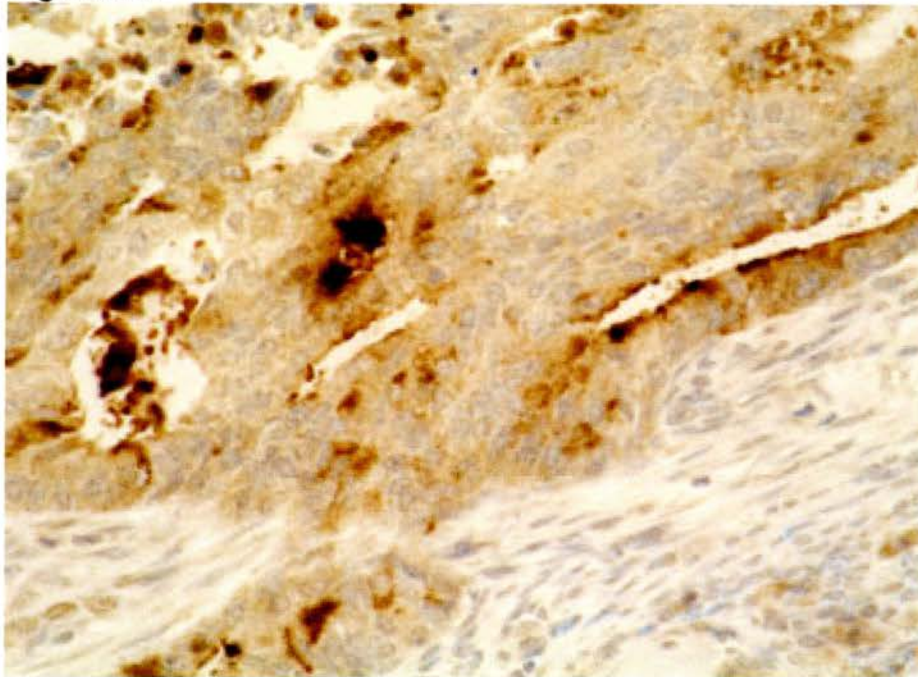


Figure 7.1. Expression of MMP-2 predominantly in UPSC stromal cells (Magnificationx20).

Figure 7.2. Expression of MMP-7 in both carcinoma and stromal cells of UPSC (Magnificationx20).

Figure 7.3

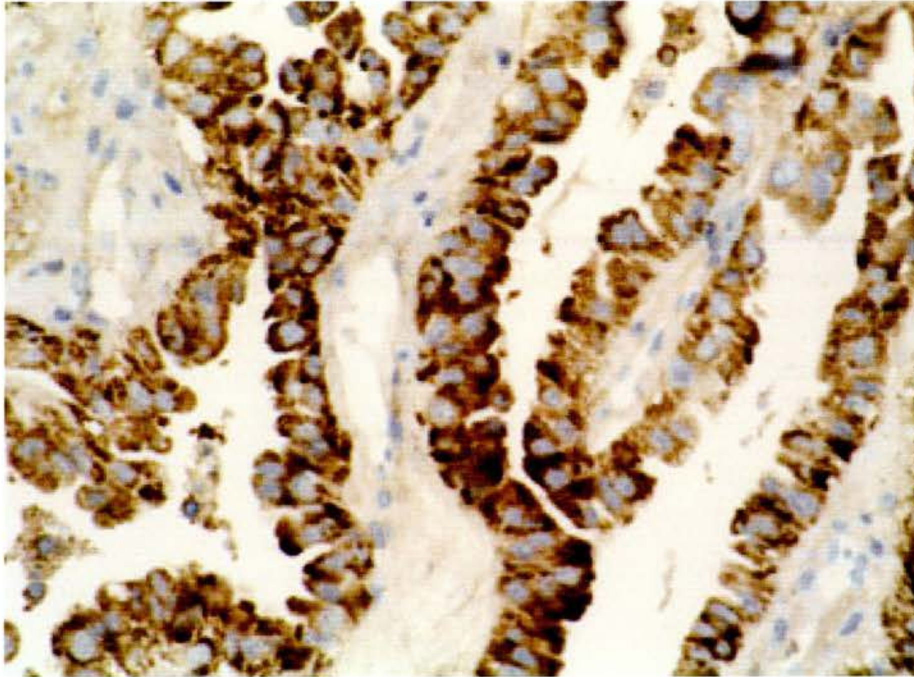


Figure 7.3. Expression of MMP-9 predominantly in UPSC carcinoma cells (Magnificationx20).

7.3.2.2 Endometrioid Endometrial Carcinoma

No significant differences in expression of any of the proteins (MMPs-2, -7 or-9) were noted in either carcinoma cells or stromal cells at the invasive edge compared to those in the viable central part of the tumour (Table 7.2). Expression of all the MMPs was both cytoplasmic and membranous (Figures 7.4, 7.5 and 7.6 respectively).

There was no significant difference in expression of MMP-2 between carcinoma and stromal cells in both the viable central part of the tumour and at the invasive edge (Table 7.4). There was significantly greater expression of MMP-7 in carcinoma cells compared with stromal cells in the viable central part of the tumour ($p = 0.018$, Wilcoxon Signed Ranks Test, Table 7.4), but there was no significant difference in expression between the cell types at the invasive edge. Significantly greater expression of MMP-9 was noted in stromal cells compared to carcinoma cells in both the viable central part of the tumour and at the invasive edge ($p = 0.011$ and $p = 0.012$ respectively, Wilcoxon Signed Ranks Test, Table 7.4).

Table 7.4. Variation in Matrix Metalloproteinase Expression between Tumour and Stromal cells in Endometrioid Endometrial Carcinoma.

Antibody	Main Tumour		Invasive Edge	
	Tumour cells	Stromal cells	Tumour cells	Stromal cells
MMP-2	1.43 SD 0.97	1.42 SD 0.52	1.69 SD 0.58	1.46 SD 0.94
MMP-7	1.77* SD 0.48	1.52* SD 0.43	1.61 SD 0.57	1.51 SD 0.56
MMP-9	1.14** SD 0.89	2.51** SD 1.08	1.09^{+} SD 0.96	1.76^{+} SD 0.72

SD Standard Deviation

*P = 0.018 (Wilcoxon Signed Ranks Test)

$^{+}$P = 0.012 (Wilcoxon Signed Ranks Test)

**P = 0.011 (Wilcoxon Signed Ranks Test)

Figure 7.4

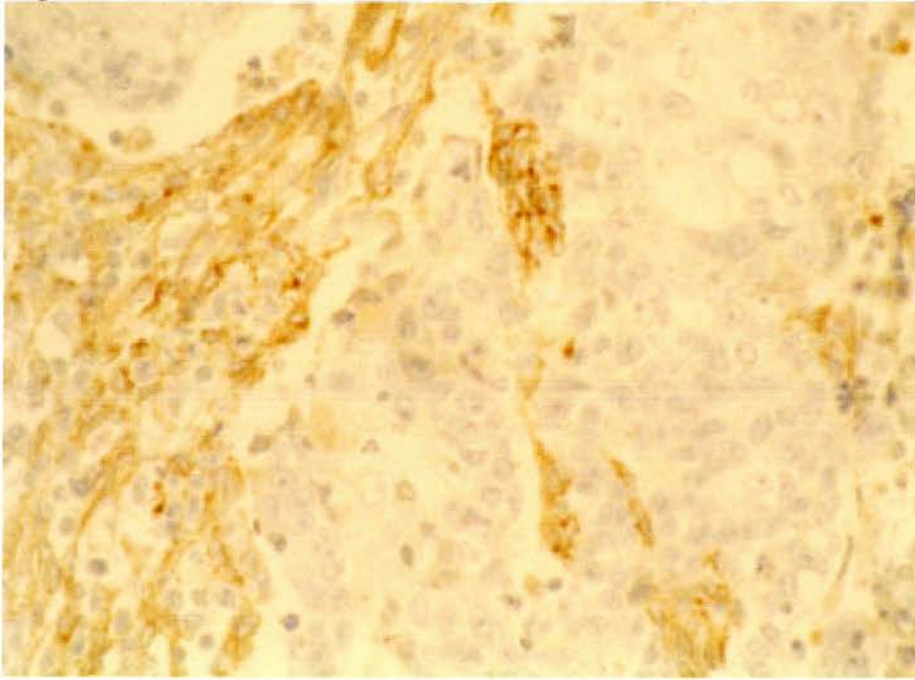


Figure 7.5

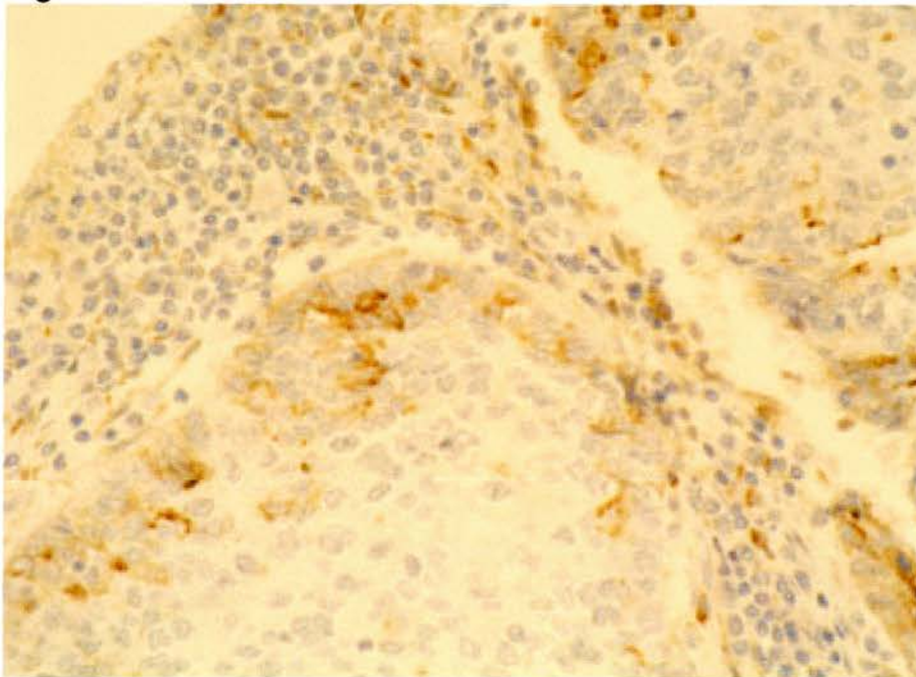


Figure 7.4. Expression of MMP-2 predominantly in EEC stromal cells (Magnificationx40).

Figure 7.5. Expression of MMP-7 in both carcinoma and stromal cells of EEC (Magnificationx40).

Figure 7.6

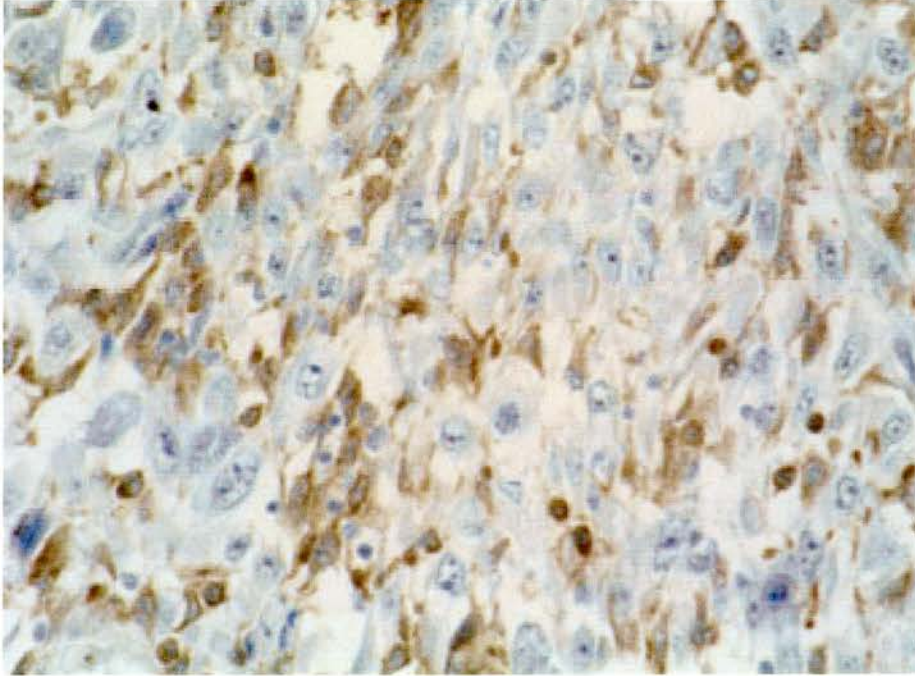


Figure 7.6. Expression of MMP-9 predominantly in EEC carcinoma cells (Magnificationx40).

7.4 Discussion

UPSC is an aggressive endometrial tumour with a notable propensity for lymphovascular space invasion and a poor prognosis. This chapter aimed to determine if MMPs had a role in the increased invasiveness of UPSC in comparison to EEC. We confirmed the findings of Misugi et al {Misugi, 2005} and Moreno-Bueno et al {Moreno-Bueno, 2003} and demonstrated MMPs -2, -7 and -9 to be present in both UPSC and EEC.

In addition, this chapter looked for differences in expression of these proteins between the different histological tumour types and within the tumours (the viable central part of the tumour and at the invasive edge). We demonstrated MMP-2 expression in both EEC and UPSC carcinoma cells, confirming the findings of Park et al {Park, 2001}. Park et al {Park, 2001} demonstrated that synthesis and secretion of stromal MMP-2 was up regulated by β oestradiol. In addition they showed that the invasiveness of cells from an endometrial adenocarcinoma cell line was enhanced by recruitment of MMP-2 secreted by endometrial stromal cells to their cell membrane, which was further enhanced by the presence of β oestradiol.

The finding of significantly higher MMP-2 expression in EEC carcinoma cells compared to those in UPSC may partly reflect the different biological properties of these tumours. EEC is a tumour which usually arises on a background of oestrogen excess, which commonly expresses ER and can produce intratumoural oestrogen using aromatase {Segawa, 2005}. On the other hand, UPSC usually arises from atrophic endometrium, is generally ER negative and does not produce intratumoural oestrogen. Park et al {Park, 2001} demonstrated that β oestradiol stimulated MMP-2

secretion in adenocarcinoma cell lines, therefore it is possible that the differential secretion may be due to increased intratumoural oestrogen in EEC compared to UPSC. We found significantly higher ER and PR positivity in our EEC cases compared to UPSC.

We found similar levels of MMP-2 in EEC carcinoma and stromal cells and although there was increased expression of MMP-2 in carcinoma cells at the invasive edge of the tumour, this was not statistically significant. However, we did find a significantly higher level of MMP-2 in UPSC stromal cells compared with carcinoma cells both in the viable central part of the tumour and at the invasive edge. In addition, the UPSC carcinoma cells showed a significant increase in MMP-2 expression at the invasive edge compared to the viable central part tumour. MMP-2 is synthesised and secreted by stromal cells and then binds to the TIMP-2:MT-MMP complex on the carcinoma cell surface {Yu, 2000}; this difference in expression raises the possibility that the invasive subclone of UPSC uses increased carcinoma cell binding of MMP-2 to equip the cells with the proteases required for invasion

We did not demonstrate any intertumoural or intratumoural differences in expression of MMP-7. There was significantly more expression of MMP-7 in carcinoma cells compared to stromal cells in both UPSC and EEC. Our findings are in keeping with the published literature that MMP-7 is generally expressed in tumours {Yabushita, 2000;Ueno, 1999;Moser, 1999;Misugi, 2005}. As this study examined high grade endometrial cancers only we could not confirm or refute the findings of Misugi et al

{Misugi, 2005}, who showed differential expression of MMP-7 between the different grades of EEC.

Crawford et al {Crawford, 1999} described a positive correlation between nuclear β catenin protein levels and MMP-7 transcripts in colonic carcinoma. They did not find any MMP-7 expression in cells that lacked β catenin protein accumulation. Mylona et al {Mylona, 2005} demonstrated an inverse correlation with nuclear β catenin expression in breast carcinoma. We did not demonstrate any inverse or direct correlation with β catenin expression and any of the MMPs. This may be due to the relatively small numbers of cases involved. Alternately this may be because the studies that demonstrated either inverse or direct correlations were focussed on colonic carcinoma where β catenin expression is nuclear, reflecting mutation in the β catenin gene and the studies by Mylona et al {Mylona, 2005} in breast carcinoma also focussed on β catenin mutation.

We found a significantly higher expression of MMP-9 in EEC compared to UPSC and also a significantly higher expression of MMP-9 in the stromal cells compared to the carcinoma cells in the viable central part of the tumour in both EEC and UPSC. In addition there was significantly higher expression of MMP-9 in stromal cells compared to carcinoma cells at the invasive edge of the EEC cases. However, there was no significant difference in MMP-9 expression between the invasive edge stromal cells and carcinoma cells in UPSC. MMP-9 is normally synthesised and secreted by stromal cells and binds to CD44 on carcinoma cells {Yu, 2000}. The loss of this differential expression between carcinoma cells and stromal cells at the

invasive edge of UPSC cases may indicate that MMP-9 synthesis and secretion by stromal cells is unchanged at the invasive edge of the tumour, but binding of MMP-9 by the invading carcinoma cells is increased, perhaps enhancing their invasive potential.

Hui et al {Hui, 2005} demonstrated differences in outcome between patients with Stage Ia UPSC arising in a polyp and in the normal endometrium. In view of our results showing differences in expression of MMPs -2 and -9 between the main part of the tumour and the invasive edge, it would be interesting to more closely examine cases of endometrial intraepithelial carcinoma and Stage Ia UPSC.

7.4.1 Summary.

In summary this immunohistochemical study has confirmed that MMPs-2, -7 and -9 are present in endometrial carcinomas and specifically in both EEC and UPSC. We also confirmed that our UPSC group had significantly lower ER and PR expression. We found significantly higher expression of MMPs -2 and -9 in EEC compared to UPSC. This may be due to the known relationship of these MMPs with oestrogen status. Despite the lower expression of MMP-2 and MMP-9 in UPSC compared to EEC we did show increased expression of MMP-2 and MMP-9 by carcinoma cells at the invasive edge of UPSC. This is probably due to increased binding of MMPs secreted by the stromal cells to carcinoma cells. This difference in staining pattern between the viable central part of the tumour and the invasive edge supports the theory of an invasive subclone of UPSC acquiring activated proteases in order to invade.

Summary of Thesis

Despite having been described as a distinct clinical entity for over twenty years, uterine papillary serous carcinoma (UPSC) remains a management challenge for the multidisciplinary team due to the paucity of understanding on the epidemiology, pathogenesis, natural history and optimal treatment strategies for this important and aggressive variant of endometrial adenocarcinoma. The aim of this thesis is to specifically focus on molecular changes in UPSC, which might account for its aggressive behaviour and pattern of spread and to add to the growing volume of literature on UPSC. This thesis briefly describes the current literature on UPSC and demonstrates that although in the past the microscopic and clinical similarities of UPSC to ovarian serous papillary carcinoma (OSPC) led to some clinicians and pathologists to think that these tumours were biologically similar, they are actually quite distinct. There are a wide number of adjuvant treatment options open to patients with UPSC; the majority of which have only been formally tested on patients with OSPC.

The tissue microarray (TMA) validation study (Chapter 3) shows that the results of immunohistochemical staining of TMAs made from endometrial carcinoma are equivalent to those from whole sections of the same tumour. The ideal number of cores sampled should be 2 to 3 in order to decrease the variation seen in heterogeneous tumours, and also to increase the likelihood of an informative section in those tumours, which may be focal, infiltrative or scanty when present in a pipelle specimen. In addition, this chapter demonstrates that TMA use can be extended to include other high-grade endometrial carcinomas and antibodies with uncertain

patterns of expression. Chapter 4 describes an audit of the pathological diagnosis and management of UPSC in South East Scotland. This confirms the poor prognosis of patients with UPSC and shows that in even those cases where the tumour only comprises 5% UPSC, the poor prognosis is conferred, regardless of the additional tumour type. This audit raises awareness for the need of accurate and complete surgical staging at a local level. Despite the poor response to chemoradiotherapy identified by this audit, local treatments for patients with UPSC have not altered, as patient numbers are small with a limited follow up time. This audit contributed to the increasing local, national and international awareness of the need to invest in randomised controlled trials on UPSC.

The remaining chapters focus on molecules involved in three steps associated with invasion. Chapter 5 examines the initial step in invasion where tumour cells decrease their homotypic binding in order to detach from one another and to invade. Therefore the expression of the cell adhesion molecules E cadherin, P cadherin and β catenin in UPSC is determined and this expression is compared with high-grade endometrioid endometrial carcinoma (EEC). This chapter confirms that E cadherin, P cadherin and β catenin are expressed in high-grade endometrial carcinoma and that there is differential E cadherin and P cadherin expression between UPSC and EEC. This may in part be due to the important role of oestrogen in the development and progression of EEC compared to UPSC. In addition, decreased expression of E cadherin and P cadherin between the viable central part of the tumour and the invasive edge is demonstrated.

The second step in invasion, after cells have dissociated from each other, is the attachment of tumour cells to molecules in the extracellular matrix (ECM). CD98

and Galectin-3 (Gal-3) were selected for this chapter (Chapter 6). CD98 was chosen due to the relatively recent discovery of its association with β1 integrin and Gal-3 was chosen as it has been identified as a possible ligand of CD98. This chapter confirms that CD98 and Gal-3 are present in both EEC and UPSC. There was significantly higher expression of CD98 in EEC compared to UPSC. This may be due to the known relationship of CD98 with β1 integrin and indirectly β1 integrin with ER status. Despite the lower expression of CD98 in UPSC compared to EEC there was increased expression of CD98 at the invasive edge of UPSC. This is may be due to increased requirement of invading UPSC cells for nutrients, or it may reflect cross linking of β1 integrin and thus activation of β1 integrin signalling and anchorage independent growth. There was no significant difference in Gal-3 expression between the tumour types, but Gal-3 expression did significantly decrease at the invasive edge of UPSC compared to the central viable part of the tumour. This decrease in Gal-3 expression also occurred to a non-significant level in EEC and was mirrored by a similar loss of nuclear expression of Gal-3. These findings raise the possibility that loss of nuclear expression of Gal-3 is associated with the development of invasive properties of these tumours.

The final step in invasion examined in this thesis is degradation of the ECM. Chapter 7 confirms that MMPs-2, -7 and -9 are present in high grade endometrial carcinoma. It also confirms that the UPSC group had significantly lower oestrogen receptor (ER) and progesterone receptor (PR) expression. Expression of MMPs-2 and -9 was significantly higher in EEC compared to UPSC. This may be due to the known relationship of these MMPs with oestrogen status. Despite the lower expression of MMP-2 and MMP-9 in UPSC compared to EEC, expression of MMP-2 and MMP-9

by carcinoma cells at the invasive edge of UPSC was increased. This is probably due to increased binding to carcinoma cells of MMPs secreted by the stromal cells. The difference in staining pattern of E cadherin, P cadherin, β catenin, CD98 and MMPs-2 and -9, between the viable central part of the tumour and the invasive edge supports the theory of a subclone of UPSC acquiring the properties necessary to invade.

In conclusion, this thesis has examined the role of molecules involved in some of the important steps involved in invasion; namely loss of cell-cell adhesion (E-cadherin, P cadherin and β catenin), attachment to the ECM (CD98 and Gal-3) and degradation of the ECM (MMPs -2, -7 and -9). This demonstrated differences in expression of these molecules, not only between the central viable part of the tumour and the invasive edge but also between UPSC and EEC. The presence of intratumoural variation supports the theory that tumours become increasingly heterogenous with subclones developing different properties necessary for invasion and metastasis. The intertumoural variation emphasises the influence of oestrogen and therefore the oestrogen receptor status of the tumours as E cadherin, β catenin, CD98 and MMPs -2 and -9, are either directly or indirectly altered by oestrogen and, apart from E cadherin which was decreased, the other molecules all showed higher expression in EEC compared to UPSC. The results are consistent with the important role that oestrogen status plays in the development of EEC and it is possible that the lack of responsiveness of UPSC to oestrogen plays a role in its aggressive behaviour.

These data contribute to the growing body of literature on UPSC, and addresses diagnostic and treatment uncertainties for the pathology, surgical and oncological teams.

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Appendix 1

The following publications resulted from the work of this thesis:

Faratian D, Stillie A, Busby-Earle RMC, Cowie VJ, **Monaghan H**. A review of the pathology and management of uterine papillary serous carcinoma and correlation with outcome. Int. J. Gynecol. Cancer 2006;16:972-978.

Monaghan H, Williams ARW. The pattern of CD98 expression is different between uterine serous papillary carcinoma and endometrioid endometrial carcinoma. Gynecol Oncol. 2007;104(1):264-5.

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A review of the pathology and management of uterine papillary serous carcinoma and correlation with outcome

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Abstract. Faratian D, Stillie A, Busby-Earle RMC, Cowie VJ, Monaghan H. A review of the pathology and management of uterine papillary serous carcinoma and correlation with outcome. *Int J Gynecol Cancer* 2006;**16**:972–978.

Uterine papillary serous carcinoma (UPSC) accounts for 10% of endometrial carcinomas but a higher proportion of deaths due to its aggressive nature and poor response to chemotherapy and radiotherapy. In order to add to the knowledge of UPSC in the literature and to review our local practices, we examined the pathology, medical records, and management of all cases of UPSC (67 patients) treated in South East Scotland over a 10-year period and also evaluated the prognostic significance of the percentage of UPSC in endometrial pipelle and hysterectomy specimens. Although only 63% of initial diagnostic biopsies were reported to contain UPSC, rereview of the cases revealed UPSC in 98.5% of the preoperative biopsies. The percentage of UPSC in the tumors did not affect the outcome. Stage, positive omentum, and treatment with external-beam +/− intracavitary radiotherapy were significantly correlated with overall survival and progression-free survival by univariate analysis, but only stage ($P < 0.01$) was correlated with outcome on multivariate analysis. Chemotherapy did not affect outcome. UPSC may be difficult to diagnose in preoperative biopsies, particularly when present as part of a mixed tumor. Even a small percentage of UPSC in a diagnostic biopsy or hysterectomy specimen is correlated with a poor prognosis. This study emphasizes the need of a cooperative, prospective study on this distinct uterine carcinoma.

KEYWORDS: chemotherapy, papillary, radiotherapy, serous, uterus.

While papillary morphology has been recognized in endometrial carcinoma since the turn of the past century, uterine papillary serous carcinoma (UPSC) was first described as a distinct clinical entity in 1982 by Lauchlan[1] and Hendrickson *et al.*[2,3] UPSC usually occurs in elderly, postmenopausal women and, in contrast to endometrioid endometrial carcinoma (EEC), is not associated with excess estrogen. There is conflicting data regarding the association of breast cancer with high-risk endometrial carcinomas[4,5]. It is thought that patients with breast cancer have a similar rate of low- and high-risk endometrial subtypes irrespective of tamoxifen use. However, patients with UPSC may have an increased risk of synchronous or subsequent development of breast cancer[6,7]. This raises the possibility that UPSC and breast cancer may, in some patients, be due to the presence of mutations in cancer predisposing genes such as BRCA 1[8].

Although accounting for only 10% of endometrial carcinomas, UPSC is an important diagnosis to make on initial biopsy since it behaves particularly aggressively, with a propensity for early metastasis that results in upstaging at the time of operation in 50–75% of clinical stage I cancers[9–11]. This underscores the need for accurate preoperative diagnosis, so that the surgeon can plan definitive treatment and perform accurate and thorough surgical staging by the FIGO

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system, including a total extrafascial hysterectomy and bilateral salpingo-oophorectomy, peritoneal washings, and removal of any suspicious pelvic or para-aortic lymph nodes. Although omentectomy is not in the 1988 FIGO system for staging, it is also frequently performed. This is because UPSC behaves aggressively and has a pattern of spread similar to that of ovarian serous carcinoma. There is no evidence to date that this radical surgery affects outcome, but accurate staging may help influence current advice regarding adjuvant therapy. UPSC may exist in both "pure" and "mixed" forms with other endometrial cancer subtypes, in particular EEC and clear cell endometrial carcinoma (CCEC). Sherman *et al.*[12] state that the diagnosis of UPSC should be reserved for cancers with more than 25% UPSC pattern in the final resection specimen, although this figure appears arbitrary.

Data as to the optimum management of UPSC are limited due to its relative rarity. This audit was performed to look at the patient characteristics, histopathologic characteristics, and outcomes of various treatment regimens of UPSC. In particular, we wished to determine the accuracy of diagnosing UPSC on endometrial pipelle biopsies or curettings, whether the histologic subtypes seen in the diagnostic biopsy correlated with those seen in the final resection specimen, and also what percentage of UPSC pattern is required to confer the poor prognostic phenotype. To date, there have been several studies looking at either the oncologic or the surgical management of UPSC, ranging from 9 to 129 cases[3,6,9,13–18]. Our review of the management of this challenging disease is the fourth largest of its kind (67 patients), and to our knowledge, the first to address these specific pathologic questions.

Materials and methods

Between 1994 and 2003, 67 patients had a tissue diagnosis of pure UPSC or a mixed carcinoma containing a UPSC component made on initial biopsy or in the resection specimen. All these cases were reviewed, and the management was discussed at the regional gynecological oncology clinicopathologic meeting. Of these, 59 patients had surgical intervention, and 57 received adjuvant therapy of radiotherapy +/− chemotherapy.

The patient demographics, histopathology, and treatment were recorded.

Patient characteristics

Surgical and oncologic medical records were reviewed to obtain information about patients' age at presentation, any previous history of breast cancer, the treatment, and follow-up data, in particular the site of any relapse. The primary end points were progression-free survival (PFS) and overall survival (OS), defined as the period from the date of diagnosis to the date of recurrence or the last clinic visit (if alive) or the date of death. Data were censored if information on survival was not available or the patient had ceased to be followed up for any reason but had not died of cancer.

Histopathologic characteristics

All diagnostic biopsies and hysterectomy sections from the 67 patients were reviewed by two pathologists (D.F. and H.M.). The diagnosis of UPSC, CCEC, and EEC were made using the classical microscopic features, which are well known. The pipelle or endometrial curettings were reviewed to determine if definitive diagnosis of UPSC was possible on the original biopsy. Then, the hysterectomy specimens were reviewed to determine if it was a pure UPSC or mixed tumor. If it was a mixed tumor, the percentage of UPSC, EEC, and CCEC of the total tumor present was estimated in the tissue processed for histologic analysis. Estimates were made by counting low-power (×20–×40) fields of each pattern and dividing this by the total number of fields. Data were compared, and the three discrepancies resolved by case conference. The number and nature of specimens additional to the hysterectomy specimen received (ie, omentum, pelvic lymph nodes, or peritoneal washings) were noted, along with the pathologic stage according to the 1988 FIGO surgical staging criteria at the time of resection.

Oncologic characteristics

The adjuvant therapy was recorded, and the chemotherapy and radiotherapy regimens (external beam +/− vaginal cesium) were documented. Where the data were available, details of the treatment of recurrence were also noted.

Statistical analysis

All statistical analyses were performed using SPSS software (SPSS Inc, Chicago, IL). The Spearman's rank test was used to assess the correlation of the initial diagnostic biopsy with the definitive hysterectomy histology percentage histologic subtype. OS and PFS were estimated on the basis of Kaplan–Meier curves and the log-rank test used as a univariate analysis of prognostic factors. A P value of less than 0.05 was considered statistically significant, and variables that had a P value of less than 0.05 were entered into a Cox regression model.

Results

Patient characteristics

The median age of the patients was 68 years (range 49–89 years). Nine patients (13%) had a medical history of breast cancer. No patients developed breast cancer after the time of diagnosis of UPSC. The median follow-up for all patients was 14.5 months (range 1–114 months). The numbers and percentages of each patient with each stage of disease are shown in Table 1, along with 1-year and 3-year OS and PFS by stage. OS and PFS correlated significantly with the stage of disease ($P < 0.01$; Fig. 1), the median OS was 67, 43, 14, and 9 months for stage I, II, III, and IV disease, respectively. The median PFS for stage I disease was not reached in this study, due to censoring, but the median PFS for stage II, III, and IV disease was 43, 9, and 8 months, respectively.

Surgical analysis

Fifty-nine patients underwent surgery. All these patients underwent a total abdominal hysterectomy, bilateral salpingo-oophorectomy. Twenty-four patients (40.1%) had, in addition, omental sampling, 21 patients (87.5%) an omental biopsy, and 3 patients (12.5%) full omentectomy. Seven patients (11.9%) had a pelvic node clearance; in 30 patients (50.9%), peritoneal washings were taken. In 7 patients total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and pelvic node sampling were carried out and peritoneal washings were taken. No patients had para-aortic node sampling.

Table 1. PFS and OS by stage

Stage	Number of patients (%)	PFS		OS	
		1 year	3 years	1 year	3 years
Stage I	25 (37)	84.7	68.2*	88.2	64.1**
IA	3 (4.5)				
IB	14 (21)				
IC	8 (12)				
Stage II	14 (20)	81.8	60.1*	81.8	50.9**
IIA	7 (10)				
IIB	7 (10)				
Stage III	19 (28)	41.7	25.0*	56.2	21.4**
IIIA	14 (21)				
IIIB	3 (4.5)				
IIIC	2 (3)				
Stage IV	9 (13)	0	ND	41.6	ND
All patients	67	61.5	46.2	67.0	39.4

ND, no data.
*$P < 0.01$; **$P < 0.01$.

Seven (29%) omental samples were positive for metastatic disease, two (28.5%) pelvic lymph node clearances were positive, while six of 30 (20%) of peritoneal washings contained malignant cells. Patients who had a negative omental biopsy at the time of operation showed a significantly better PFS and OS ($P < 0.01$) than those who had a positive biopsy (Fig. 2A). Of the three omentectomies performed, one was positive and two were negative. Positive peritoneal cytology was weakly associated with poor prognosis (log-rank test, $P = 0.15$), while the effect of pelvic lymphadenectomy on prognosis could not be analyzed due to small numbers of patients.

Histopathologic analysis

On reviewing the pathology reports, 63 of the cases were diagnosed by pipelle biopsy, curettage, or hysteroscopic biopsy; one case was diagnosed on cervical cytology, and three cases were from hysterectomy histology. Of the 63 patients who had a preoperative diagnostic biopsy, 23 biopsies (36.5%) showed pure UPSC histology and 39 (62%) showed mixed histologic subtypes including a UPSC component. Thirty nine of the diagnostic biopsies (63%) were described as serous, papillary, mixed serous/other histologic subtype in the original pathologic reports, and on review, 62 of the biopsies (98.5%) contained a UPSC component. The one biopsy (1.5%) that did not contain a UPSC component was reviewed as 100% CCEC, and on hysterectomy, the tumor was reviewed as 100% UPSC. The correlation between the initial biopsy and the hysterectomy histology subtypes is illustrated in Figure 3. While there was a weak positive correlation between the diagnostic biopsies and the hysterectomy diagnoses for UPSC and CCEC (Spearman's rank correlation coefficient 0.47 and 0.30, respectively, $P < 0.01$), the correlation is much stronger for EEC (Spearman's rank correlation coefficient 0.70, $P < 0.01$). In addition, those biopsies, which overestimated the amount of UPSC in the resection, were usually the same cases in which the percentage of CCEC was underestimated. In Kaplan–Meier analyses of survival, the OS and PFS were not significantly different in patients with pure and mixed histologic patterns or percentage of UPSC (including less than 25% UPSC compared to more than 25% UPSC) in the diagnostic biopsies or the resections (Fig. 4). In addition, whether UPSC was mixed with EEC or CCEC made no difference to survival. Serous endometrial intraepithelial carcinoma was identified in two of the hysterectomy specimens.

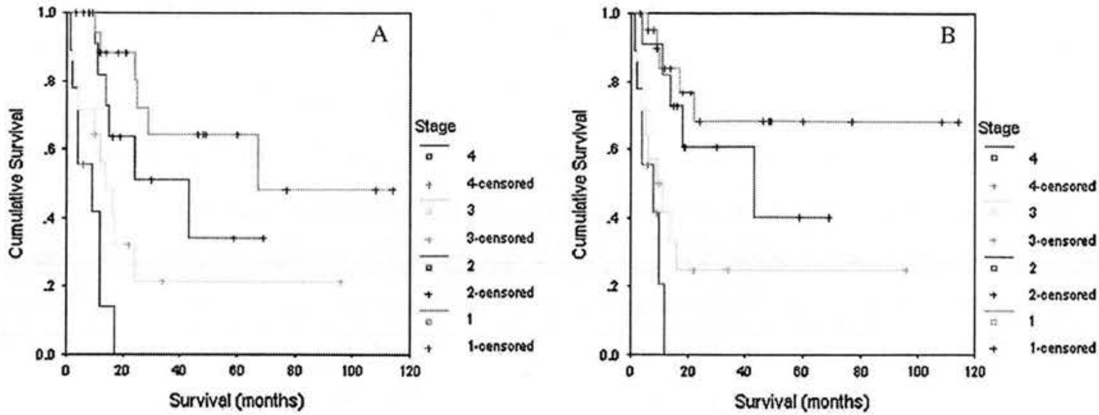

Figure 1. A) OS by stage ($P < 0.01$), and B) PFS by stage ($P < 0.01$).

Adjuvant therapy

Fifty-seven patients received adjuvant therapy. Thirty patients (52.6%) received radical radiotherapy, and three patients (5.2%) palliative therapy. Of the two patients who did not receive radiotherapy, one was deemed to have had curative surgery (stage I disease) and the other was deemed unfit for therapy. In addition to radiotherapy, 28 patients (49.1%) received adjuvant chemotherapy with different regimens including carboplatin alone, carboplatin and paclitaxel, or

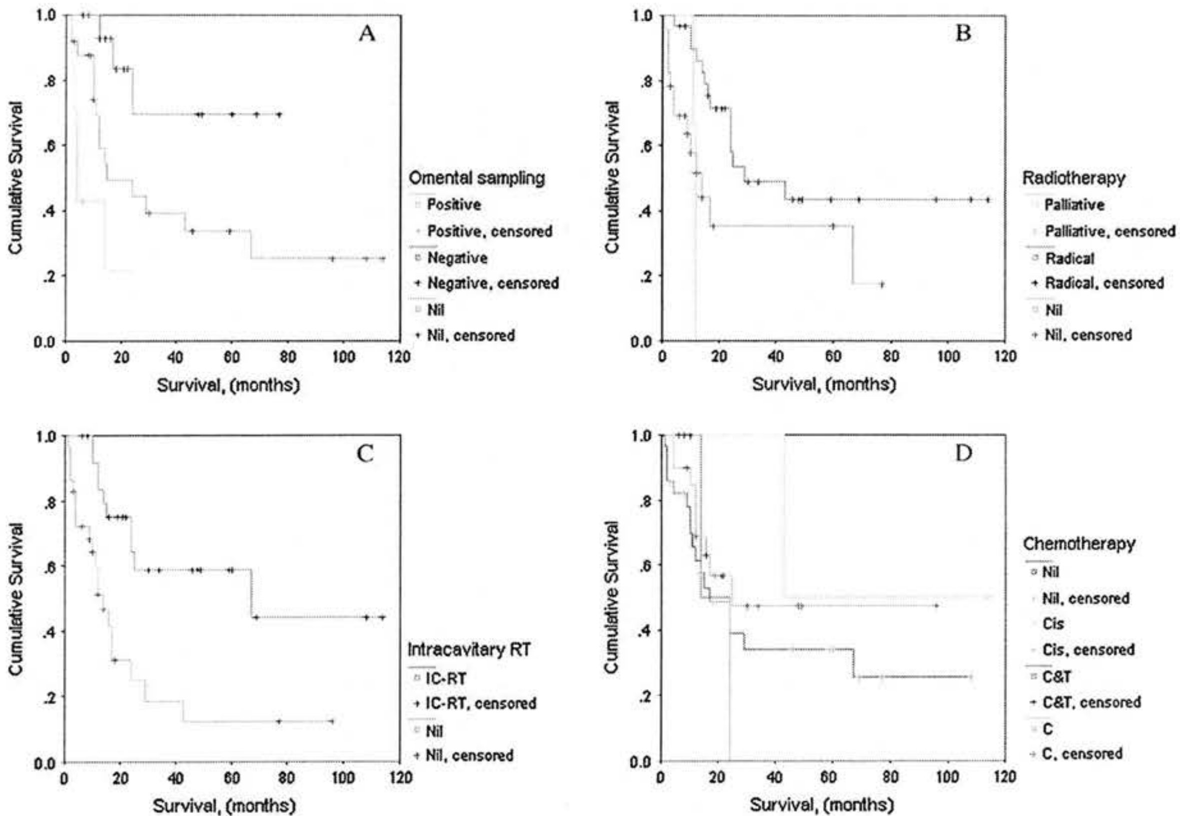

Figure 2. Kaplan–Meier curves of treatment variables by OS. A) Omental sampling $P < 0.01$ (log-rank test), B) radiotherapy $P < 0.01$ (log-rank test), C) intracavitary radiotherapy $P < 0.01$ (log-rank test), and D) chemotherapy $P = 0.64$ (log-rank test).RT, radiotherapy; Cis, cisplatinum; C&T, carboplatin and paclitaxel (Taxol); C, carboplatin.

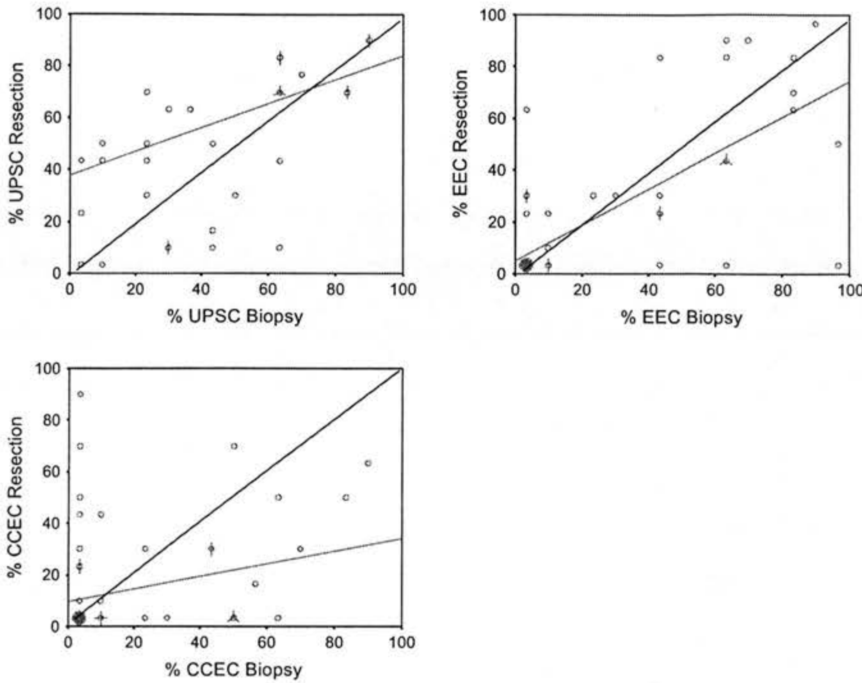

Figure 3. Scatter plots to represent percentages of histologic subtypes in diagnostic biopsies vs resection specimens. Each spoke on a data point represents one case. Best-fit lines (red) show a positive correlation between the two specimens for all three histologic subtypes.

cisplatin (Table 2). Patients treated with radical radiotherapy postoperatively had a significantly better prognosis by univariate analysis ($P < 0.01$; Fig. 2B, C). The median OS was 29 months in patients receiving radical radiotherapy compared with 12 months in patients not receiving radical radiotherapy, and the median OS was 67 months in patients receiving palliative radiotherapy compared with 12 months in patients not receiving palliative radiotherapy. There were no significant differences in survival in patients treated with or without chemotherapy (Fig. 2D) or between the different chemotherapy regimens.

Recurrence analysis

Fifteen (22.4%) patients had recorded recurrences. Of these, five (33%) were distant metastases (lung, liver, omentum, and neck lymph nodes) and ten (67%) were locoregional recurrences. Median time to recurrence was 9 months (range 4–17 months). Two omental recurrences were in patients who had positive omental biopsies. Neither had underwent complete omentectomy. The recurrence rate in stage I–IV patients was 12%, 14%, 36%, and 33%, respectively. Further survival analysis was not possible due to the small numbers in each stage and nonstandardized treatment regimens.

Discussion

This study of UPSC is the third largest retrospective series of its kind with focus on the effect of histopathology on outcome. The high median age of the patients, and postmenopausal status, is similar to that

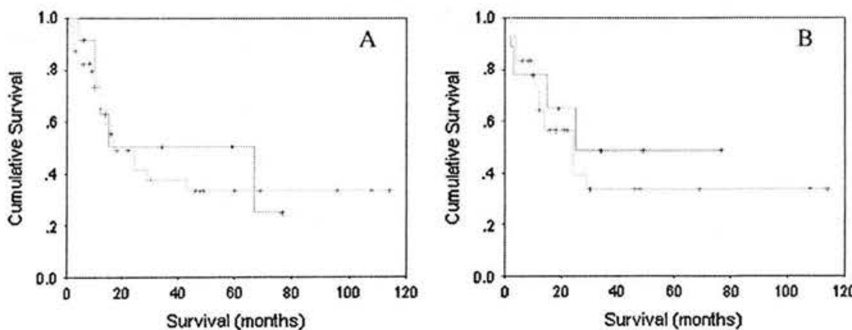

Figure 4. OS. Less than 25% UPSC (purple line) compared with greater than 25% UPSC (green line) in preoperative (A, $P = 0.76$, log-rank test) and resection specimens (B, $P = 0.56$, log-rank test).

Table 2. Chemotherapy regimens by stage

Stage	C	C&T	Cis	Nil
I	7	2	1	13
II	4	0	1	6
III	5	3	0	5
IV	5	0	0	4

C, carboplatin; Cis, cisplatinum; C&T, carboplatin and paclitaxel.

previously documented for UPSC[17,19]. Thirteen percent of patients had a personal history of breast cancer, identical to that observed by Slomovitz *et al.*[17] and similar to Barakat *et al.*[4] who observed a similar rate of high-risk endometrial subtypes in breast cancer patients regardless of tamoxifen use. The observed high proportion of UPSC in breast cancer patients appears not to be related to BRCA gene status, which confers an increased risk of ovarian papillary serous carcinoma and EEC only[6,7]. This suggests that the observed association between UPSC and breast cancer may be due to the presence of mutations in a variety of other cancer predisposing genes such as P53 and cerbB2.

Only 63% of the biopsies were diagnosed as pure or mixed UPSC histologic patterns, in line with previous reports[9,12,14,17,20–22], which have reported the accuracy of preoperative biopsy from 65% to 93%. Most biopsies not explicitly stated as UPSC were given grade 3 endometrial carcinoma diagnoses or classified according to the other major histologic subtype, usually CCEC. However, on retrospective review of the biopsies, only one (1.5%) of the preoperative biopsies did not contain any UPSC, according to traditional diagnostic criteria, and the major pattern in this case was CCEC. This may be explained by the heterogeneity of the disease.

We sought to evaluate whether 25% UPSC pattern, the value above which the tumor is classified as UPSC, is prognostically valid[11]. We found that the survival of patients with less than 25% or more than 25% UPSC in the biopsy or the resection was the same, suggesting that tumors should be classified, and managed, as UPSC regardless of the percentage of UPSC in the pathologic specimen. Our lowest percentage of UPSC used was 5%. In addition, in keeping with previous reports, pure or mixed histologic patterns did not show any survival difference, and survival was the same regardless of whether UPSC was mixed with a high-risk subtype, such as CCEC, or a low-risk subtype, such as EEC[9,17], suggesting that UPSC is biologically dominant in heterogeneous tumors.

We found that a low number of patients underwent complete surgical staging (40% omental staging, 11.9% pelvic node clearance, 50% cytology), which may in part be explained by the changing surgical practice over the period analyzed and difficulties with the interpretation of the diagnostic biopsies. While this raises the possibility that the patients might not be accurately surgically staged, our survival rates are consistent with the generally accepted long term survival rates of 35–50% for patients with stage I and II UPSC and 0–15% for patients with stage III and IV diseases[23].

Gehrig *et al.*[24] showed that the sensitivity of a visually negative omentum was 89%, concluding that, with a microscopic metastasis rate of 4%, surgical sampling does not need to be included in the routine surgical staging of UPSC. The clinical consequences of understaging is underscored by the fact that in our data two recurrences (13%) occurred in biopsy positive omenta that were not removed by omentectomy, and these two patients had stage III disease that, while still a poor prognosis group, may have benefited from additional abdominal surgical clearance. Omentectomy may result in better local control of disease and more directed and appropriate adjuvant therapy. Unfortunately, our data were too small to establish a link between omentectomy and survival. Although we had a low rate of pelvic lymphadenectomy, the diagnostic value of systematic lymphadenectomy has recently been validated by Slomovitz *et al.*[17], who reported 19% lymph node involvement in patients without myometrial invasion. Furthermore, the importance of performing a formal lymph node dissection is emphasized by the absence of pelvic sidewall failures following observation in a population at risk for recurrence[25] and may explain why two of the patients, who had not underwent formal pelvic lymphadenectomy, had pelvic recurrences.

We did not show any significant impact of adjuvant radiotherapy or chemotherapy on PFS or OS. Ramondetta *et al.*[26] showed that single-agent paclitaxel showed a tumor response in 77% of patients and used in a neoadjuvant fashion with cisplatin, and Zanotti *et al.*[27] observed responses in eight out of nine patients. In addition, Kelly *et al.*[22] recently showed that platinum-based chemotherapy improved disease-free survival and OS of patients with stage I UPSC and vaginal cuff radiation provided local control of disease. However, our study failed to show that treatment with these agents converted into an objective improvement in PFS or OS. This is probably due to the short average follow-up time (14 months) and possibly the inadequate surgical staging, resulting in patients being understaged.

Twenty-two percent of patients in this study had further disease. This is far fewer than the percentage

observed by other groups, who have documented recurrence rates of between 50% and 75%, using various adjuvant therapies, radiotherapy alone, or with the addition of chemotherapy using platinum and/or paclitaxel alone, as used in our study[27–30]. Our results, therefore, compare favorably with similarly staged tumors at equivalent median follow-up, although similar to these studies stage III and IV tumors remain a particular management problem due to higher rates of recurrence (36% and 33% for stage III and IV carcinomas compared with 12% and 14% for stage I and II carcinomas, respectively). Establishing the exact reasons for favorable recurrence rates is hampered by limited patient numbers and inconsistent and nonrandomized adjuvant therapies, but it appears that surgery produces good regional control in this group, with 67% locoregional control compared with the 87% observed by Sood *et al.*[28].

Despite having been described as a distinct clinical entity for over 20 years, UPSC remains a management challenge for the multidisciplinary team due to the paucity of understanding on the epidemiology, pathogenesis, natural history, and optimal treatment strategies for this important and aggressive variant of endometrial adenocarcinoma. These data contribute to the growing body of literature on UPSC and address diagnostic and treatment uncertainties for the pathology, surgical, and oncologic teams. This study raised awareness for the need of accurate and complete surgical staging at a local level. However, we have not altered our approach to the use of adjuvant chemoradiotherapy for patients with UPSC as our patient numbers were small with a limited follow-up time. This study contributes to the increasing local, national, and international awareness of the need to invest in randomized clinical research trials on UPSC, at a time where alternative treatment modalities may become increasingly effective.

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Letter to the Editor

The pattern of CD98 expression is different between uterine serous papillary carcinoma and endometrioid endometrial carcinoma

To the Editor:

CD98 is a disulphide-linked 125-kDa heterodimeric membrane glycoprotein, which is found on the cell surface of most normal cells, and accumulating evidence suggests that it may have a role in tumour progression and metastasis. We have investigated the expression of CD98 in uterine serous papillary carcinoma (UPSC) and endometrioid endometrial carcinoma (EEC), tumour types we selected for study because of differing pathogeneses and patterns of local invasion and metastasis.

CD98 has been shown to be involved in cellular proliferation, cell transformation, cell fusion, cell adhesion and the L-type amino acid transporter (LAT) system, in addition to regulating integrin activation, and therefore integrin signalling and anchorage independent growth [1]. Its expression is upregulated in a variety of tumours including those of larynx, where a diffuse pattern of expression is associated with more extensive disease and poorer differentiation [2]. A recent study showed down-regulation of gene expression of CD98 in cell lines derived from metastatic adenoid cystic carcinoma compared to cell lines created from primary adenoid cystic carcinomas [3]. To date, no studies have been published on the pattern of expression of CD98 in endometrial carcinomas.

Endometrial carcinoma is the most common pelvic genital malignancy in the western world. The most common subtype is EEC, which accounts for about 80% and UPSC accounts for approximately 10% of endometrial carcinomas. UPSC is associated with lymphovascular invasion and widespread dissemination and has a significantly poorer prognosis at Stages 1, 2 and 3 compared to EEC.

This study was undertaken to determine whether there is differential expression of CD98 between EEC and UPSC, which might explain the increased aggressiveness of UPSC compared to EEC. We examined levels of expression not only in the central viable part, but also at the invasive edge of these tumours.

We created tissue microarrays (TMAs) from 73 cases of UPSC and 20 cases of grade 3 EEC using the established technique [4]. At least three cores were taken from the viable central part and from the invasive edge of the tumours. Immunohistochemistry (IHC) was performed for CD98 (Primary polyclonal IgG antibody, Santa Cruz, dilution 1:200) with tonsil as the positive control. Negative controls were obtained by omitting the primary antibody. All slides were stained using the standard streptavidin biotin procedure for IHC. All CD98 expression was membranous and was assessed using a histoscore. This was calculated by multiplying the intensity of staining (1 = weak, 2 = moderate and 3 = strong) by the percentage of positively stained cells (1 = <20%, 2 = 20–80% and 3 = >80%), thus giving a score between 1 and 9. To minimise erroneous results, the average score of the cores sampled from each case was used in calculations.

Table 1 shows a summary of the results. Both tumour types showed specific staining of neoplastic cells, but there was significantly greater CD98 expression in EEC compared to UPSC. The invasive edge of UPSC showed significantly greater expression of CD98 compared to the viable central part of the tumour, whereas in EEC there was a tendency (which did not achieve significance) for expression to be decreased at the invasive edge.

These findings may reflect the different biological properties of these tumours. EEC usually arises on a background of oestrogen excess, commonly expresses oestrogen receptors (ER), and can produce intratumoural oestrogen using aromatase. In contrast, UPSC usually arises from atrophic endometrium, is generally ER negative and does not produce intratumoural oestrogen. Lessey et al. [5] found that integrin expression correlated with steroid receptor status, as well as with grade, stage and depth of invasion. We found significantly higher ER and progesterone receptors (PR) positivity in our EEC cases compared to UPSC (results not shown).

Our finding of increased expression in the viable central part of EEC and a non-significant decrease at the invasive edge is similar to that described by Huang et al. in adenoid cystic carcinoma [3]. In

Table 1
Expression of CD98 in the central part of the tumour and at the invasive edge

CD98 (Primary polyclonal IgG antibody, Santa Cruz, dilution 1:200)	UPSC		EEC	
	Tumour	Invasive edge	Tumour	Invasive edge
	*4.32 †	5.10†	*5.94	3.92

* $P = 0.009$ (Mann–Whitney U test).
† $P = 0.005$ (Wilcoxon Signed Ranks).

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contrast, lower levels of CD98 were seen in the viable central part of UPSC when compared to the invasive edge. This raises the possibility that CD98 has different roles in EEC compared to UPSC. The high CD98 in the viable central part of EEC may be due in part to the relative increase in PR and ER, but it is also possible that CD98 plays a role in increasing nutrient uptake in proliferating cells via the LAT system. In contrast, the increased expression of CD98 at the invasive edge of UPSC compared to the viable central part may reflect activation of the β1 integrin signalling pathway, increased anchorage independent growth and thus invasion.

We have shown differences in expression of CD98 between EEC and UPSC, which may reflect the different biological properties of these tumours. Further investigation of CD98 interactions with cell adhesion molecules may cast light on mechanisms of tumour invasiveness and metastasis.

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The role of matrix metalloproteinases-2, -7 and -9 and β-catenin in high grade endometrial carcinoma

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The role of matrix metalloproteinases-2, -7 and -9 and β-catenin in high grade endometrial carcinoma

Aims: To determine the expression of matrix metalloproteinases (MMPs)-2, -7 and -9 and β-catenin in uterine serous carcinoma (USC) and endometrioid endometrial carcinoma (EEC) and to investigate any difference in expression between EEC and USC which might explain the mechanism of invasion and aggressive behaviour of USC.
Methods and results: Tissue microarrays were created from the viable central part and from the invasive edge of 20 cases of grade 3 EEC and 73 cases of USC. Immunohistochemistry was performed using antibodies to MMPs-2, -7 and -9 and β-catenin. MMPs-2, -7 and -9 and β-catenin were present in both tumour types; there was significantly higher expression of MMPs-2 and -9 in EEC compared with USC and significantly increased expression of MMPs 2 and 9 by carcinoma cells at the invasive edge of USC.
Conclusions: MMPs-2, -7 and -9 and β-catenin are present in EEC and USC. The increased expression of MMPs-2 and -9 by carcinoma cells at the invasive edge of USC is possibly due to increased binding of MMPs secreted by the stromal cells to carcinoma cells, thus equipping the USC carcinoma cells with proteases for invasion.

Keywords: β-catenin, endometrial carcinoma, matrix metalloproteinases, uterine serous carcinoma

Abbreviations: BM, basement membrane; ECM, extracellular matrix; EEC, endometrioid endometrial carcinoma; MMP, matrix metalloproteinase; TMA, tissue microarray; USC, uterine serous carcinoma

Introduction

Endometrial carcinoma is the most common female genital malignancy in the western world.[1] There are two main types of endometrial carcinoma: endometrioid endometrial carcinoma (EEC) (Type I) and non-endometrioid endometrial carcinoma (Type II), which mainly consists of uterine serous carcinoma (USC), clear cell carcinoma and malignant mixed mesodermal tumour (carcinosarcoma). EEC accounts for approximately 80% of all endometrial carcinomas and usually arises from atypical hyperplasia of the endometrium.[2–4] It is associated with oestrogen excess. USC accounts for approximately 10% of cases of endometrial carcinoma[2] and makes up the majority of the Type II carcinomas. It is an aggressive tumour, usually occurring in elderly women and is thought to arise from endometrial intraepithelial carcinoma.[5–7] It is not associated with either atypical hyperplasia or a hyperoestrogenic state. Although USC comprises 10% of cases of endometrial carcinoma,[2] it accounts for a much smaller proportion of Stage I endometrial carcinomas.[8–10] It is upstaged at the time of surgery in 60% of cases.[11–15] Hui *et al.*[16] examined a series of 40 cases of minimal uterine serous carcinoma. Nine of these were endometrial intraepithelial carcinoma and 31 were cases of Stage 1a USC. They demonstrated that only when the carcinoma was confined to an endometrial polyp did the patients have good prognosis. This aggressive behaviour raises the possibility that USC interacts with the extracellular matrix (ECM) in a different way from EEC.

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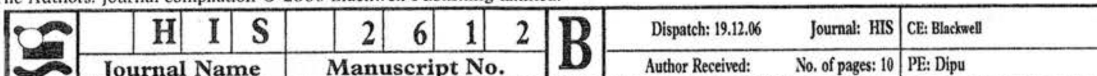

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Invasion and metastasis of tumour cells requires a variety of complex interactions including cell–cell interactions, cell–matrix interactions and degradation of the ECM. Basement membranes (BMs) constitute the major natural barrier that cancer cells must cross several times during the metastatic cascade. Liotta *et al.*[17] have suggested a hypothetical model in which tumour invasion and metastasis results from repetition of three steps: adhesion of cancer cells to BM glycoproteins; degradation of BMs by specific proteolytic enzymes; and migration of cancer cells.

The theory of tumour progression and heterogeneity is well established. Tumours are known to lose clonality early in development and generate subclones with varying characteristics resulting from mutations. In theory, only the selected subclone will possess the appropriate genetic characteristics in order to invade and metastasize.

Matrix metalloproteinases (MMPs), a family of zinc-dependent endoproteinases, are widely accepted to play a role in the invasion and metastasis of tumours.[18,19] Under normal physiological conditions, MMPs are expressed at a very low level in adult tissues, except in tissues that undergo remodelling such as cycling endometrium, normal breast tissue at the time of involution and the skin during wound healing.[18,20–22] Increased MMP expression and proteolytic degradation of ECM have been detected in a wide range of cancers, including those arising from the breast, colon, ovary and lung and MMP expression has been correlated with primary tumour growth and angiogenesis and also tumour invasion and metastasis.[18,19,23–27]

Although some MMPs are synthesized and secreted by cancer cells, others are synthesized and secreted by stromal cells and then bind to the cell membrane of tumour cells.[28–34] MMP expression appears to vary among host organ microenvironments and stromal MMPs may promote metastasis to one organ in preference to another. Therefore, antimetastatic effects based on MMP inhibition may be dependent on MMPs derived from specific organ microenvironments as well as tumour cells.[35]

ProMMP-2, the precursor to active MMP-2, is widely expressed in normal tissue and the *MMP-2* gene has been described as a 'house-keeping' gene for its role in normal cellular processes.[36] In contrast, active MMP-2 is increased significantly in some neoplastic tissues and absent in most normal tissues. MMP-2 contributes to cell migration by a mechanism involving interaction with collagen.[37] MMP-2 has been demonstrated in endometrial carcinoma cells.[38]

Altered expression of MMP-7 has been demonstrated in EEC and increased expression of MMP-7 is associated with lymph node metastasis.[38–41] Matrisian *et al.*[36] have shown that induction of MMP-7 expression in tumour cells increases tumorigenicity and metastases, while inhibition of MMP-7 leads to decreased metastasis.[42,43] In addition, they found that MMP-7 expression appeared to be associated with a less aggressive phenotype, despite being involved in invasion. In contrast, Misugi *et al.*[38] have demonstrated that increased expression of MMP-7 is found in high-grade endometrial carcinomas.

β-Catenin has roles in both cell–cell adhesion and intracellular signalling. It links to intracellular E-cadherin and thus connects E-cadherin to the actin cytoskeleton.[44–46] Cadherin–catenin complexes also have roles in intercellular communication and modulation of cell function in both normal and malignant tissues. Failure to assemble the E-cadherin–catenin complex or to connect properly to the actin cytoskeleton results in loss of cell adhesion, which may be involved in tumour spread. Signalling through the cadherin–catenin complexes is involved in the regulation of epidermal growth factor receptor distribution.[47]

MMP-9 expression has been shown to be altered in EEC and increased expression is associated with myometrial invasion.[38–41] In addition, Park *et al.*[48] have shown that synthesis and secretion of MMP-9 is down-regulated by β-oestradiol. Cioppi *et al.*[49] found that MMP-9 expression is higher in oestrogen receptor-positive endometrial carcinomas.

Although USC comprises only 10% of endometrial carcinomas, it accounts for a greater proportion of deaths. This study was undertaken to investigate the expression of MMPs-2, -7 and -9 in USC and EEC, and in particular to see if there was any difference in expression between the two tumour types which might explain the observed difference in aggressive behaviour. As β-catenin has a role in cell–cell adhesion and studies on colon and breast carcinoma have shown both positive and negative correlations between the expression of β-catenin and MMP-7, we also examined the expression of β-catenin in these tumours. It is well recognized that tumours are heterogeneous and examining the central part of the tumour alone may give misleading results as regards the invasiveness of the tumour as a whole. We used tissue microarrays (TMAs) to examine a relatively large number of cases, in order to be able to demonstrate differences in expression of these proteins between the viable central part of the tumour and the invasive edge in USC and EEC.

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The Medicine/Clinical Oncology II Research Ethics Committee of the Lothian Research Ethics Committee gave ethical approval for this study.

Materials and methods

TISSUES

Archival tissue samples were selected from the Royal Infirmary of Edinburgh Department of Pathology, the slides were reviewed by H.M. and A.R.W.W. the diagnosis of USC or EEC was confirmed and appropriate blocks selected. Haematoxylin and eosin (H&E) sections then had at least one area from the centre of the tumour and invasive edge marked on the slide by the pathologist. The samples included 20 International Federation of Gynaecology and Obstetrics (FIGO) Grade 3 cases of EEC and 73 cases of USC. As we did not identify any cases of endometrial intraepithelial carcinoma and only three cases of USC were at Stage 1a, these cases were not treated as a separate group. All tissue had been routinely fixed in 4% buffered formaldehyde and processed to paraffin blocks by routine methods.

TISSUE MICROARRAY

A microarray instrument (Beecher Instruments, Sun Prairie, WI, USA) was used. In order to construct the microarray, empty paraffin blocks of a depth of 5–10 mm were produced. Cores of wax, 0.8 mm diameter, were extracted from the empty blocks and replaced with cores of 0.6 mm diameter taken from the tissue blocks at sites corresponding to the previously selected areas on the H&E slides. Three cores were taken from each area marked on the slide, so that at least three cores were taken from each case. The cores were punched at 1-mm intervals, at least three cores per case, to decrease the risk of aberrant results due to tumour heterogeneity. A grid system with each core having a coordinate reference (X axis, Y axis) was used to allow cross reference between core location and parent case. The microarray blocks were identified as EEC tumour, EEC invasive edge or USC tumour or USC invasive edge.

Once the microarrays were complete, the blocks were sealed in a 60°C oven for 10 min.

IMMUNOHISTOCHEMISTRY

From the array blocks, sections were cut at 3 μm thickness and mounted on positively charged capillary action slides (Dako, Copenhagen, Denmark) and incubated at 60°C overnight. The slides were dewaxed and rehydrated prior to antigen retrieval.

MMP-2

Antigen retrieval was performed by immersing the test slides along with a section of normal appendix (as the positive control) in 0.01 M ethylenediamine tetraaceticacid (EDTA), pH 8.0 and microwaving at high power for 15 min. The primary monoclonal antibody was the 17B11 clone (NovoCastra Laboratories, Newcastle, UK) and was used at a dilution of 1 : 40. Negative controls were obtained by omitting the primary antibody.

MMP-7

No antigen retrieval was performed. A slide of breast carcinoma was used as the positive control. The primary monoclonal antibody was clone 1D2 (Chemicon International, Chandlers Ford, UK) and was used at a dilution of 1 : 25. Negative controls were obtained by omitting the primary antibody.

MMP-9

Antigen retrieval was performed by immersing the test slides along with a slide of kidney (as the positive control) in 0.01 M EDTA, pH 8.0 and pressure cooking for 7 min. The primary monoclonal antibody was clone 15W2 (NovoCastra Laboratories) and was used at a dilution of 1 : 80. Negative controls were obtained by omitting the primary antibody.

β-CATENIN

Antigen retrieval was performed by immersing the test slides along with a slide of tonsil (as the positive control) in 0.01 M EDTA, pH 8.0 and microwaving at high power for 15 min. The primary monoclonal antibody was the β-catenin-1 clone (Dako) and was used at a dilution of 1 : 50. Negative controls were obtained by omitting the primary antibody.

All slides were stained on the Dako TechMate™ 500 plus, using the Dako ChemMate™ EnVision™ Detection kit and the standard operating procedure for routine immunohistochemistry.

SCORING

The scores for MMP-2, MMP-7, MMP-9 and β-catenin were calculated by multiplying the intensity of staining (1, weak; 2, moderate; and 3, strong) by the percentage

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of cells showing positive nuclear staining (1, < 20%; 2, 20–80%; and 3, > 80%), thus giving a score between 1 and 9.

STATISTICAL ANALYSIS

The mean scores of the three areas of tumour assessed for each case of USC and EEC were calculated. The results from the main part of the tumour were used in calculations to determine if there were any differences between tumour types. A paired *t*-test was performed for the parametric results and a Mann–Whitney *U*-test was performed for the non-parametric results. A *P*-value of < 0.05 was considered to be significant.

The Wilcoxon matched-pairs signed-ranks test was used to estimate the relationship between staining patterns of the different antibodies used between the main part of the tumour and at the invasive edge. A *P*-value of < 0.05 was considered to be significant. The Pearson pairwise correlation coefficient was used to determine any correlation between expression of different MMPs and β-catenin.

Results

INTERTUMOUR VARIABILITY

Significantly greater MMP-2 expression was present in EEC carcinoma cells compared with USC carcinoma cells ($P < 0.05$, Mann–Whitney *U*-test; Table 1). There was no significant difference in MMP-2 expression between USC and EEC stromal cells. In addition, there was no significant difference in either carcinoma cell or stromal cell expression of MMP-7 between USC and EEC. Although there was no significant difference in MMP-9 expression between USC and EEC carcinoma cells, there was significantly greater MMP-9 expression seen in EEC stromal cells ($P = 0.001$, Mann–Whitney *U*-test; Table 1). There was no significant difference in β-catenin expression between USC and EEC. Scatter-plots and Pearson pairwise correlation coefficient showed no direct or inverse correlation between β-catenin expression and MMPs-2, -7 or -9.

Table 1. Expression of matrix metalloproteinases (MMPs)-2, -7 and -9 and β-catenin in the central part of the tumour in uterine serous carcinoma (USC) and endometrioid endometrial carcinoma (EEC)

Antibody	USC	EEC
MMP-2	0.53*	1.43*
MMP-2 stroma	1.68	1.36
MMP-7	1.81	1.77
MMP-7 stroma	1.28	1.51
MMP-9	0.90	1.17
MMP-9 stroma	1.24**	2.44**
β-Catenin	7.05	7.78

*$P < 0.05$ (Mann–Whitney *U*-test); **$P = 0.001$ (Mann–Whitney *U*-test).

INTRATUMOUR VARIABILITY

Uterine serous carcinoma

There was significantly greater MMP-2 expression in carcinoma cells at the invasive edge compared with those in the viable central part of the tumour ($P = 0.045$, Wilcoxon signed-ranks test). However, there was no significant difference in expression of MMP-2 in stromal cells between the invasive edge and the viable central part of the tumour (Table 2). MMP-2 immunoreactivity was present in both the cytoplasm and at the cell membrane (Figure 1B).

There was no significant difference in either MMP-7 or MMP-9 expression between the viable central part of the tumour or the invasive edge in either carcinoma or stromal cells (Table 2). Immunoreactivity of both MMP-7 and MMP-9 in both carcinoma and stromal cells was cytoplasmic and membranous (Figures 1D and 2B, respectively).

Table 2. Expression of matrix metalloproteinases (MMPs)-2, -7 and -9 and β-catenin in the central part of the tumour and at the invasive edge

	USC		EEC	
Antibody	Tumour	Invasive edge	Tumour	Invasive edge
MMP-2	0.53	0.79*	1.43	1.69
MMP-2 stroma	1.68	1.54	1.42	1.46
MMP-7	1.81	1.69	1.77	1.61
MMP-7 stroma	1.27	1.21	1.52	1.51
MMP-9	0.90	1.08	1.14	1.09
MMP-9 stroma	1.24	1.22	2.51	1.76
β-Catenin	7.05	7.94**	7.19	7.63

*$P = 0.045$ (Wilcoxon signed-ranks test); **$P = 0.03$ (Wilcoxon signed-ranks test).

USC, Uterine serous carcinoma; EEC, endometrioid endometrial carcinoma.

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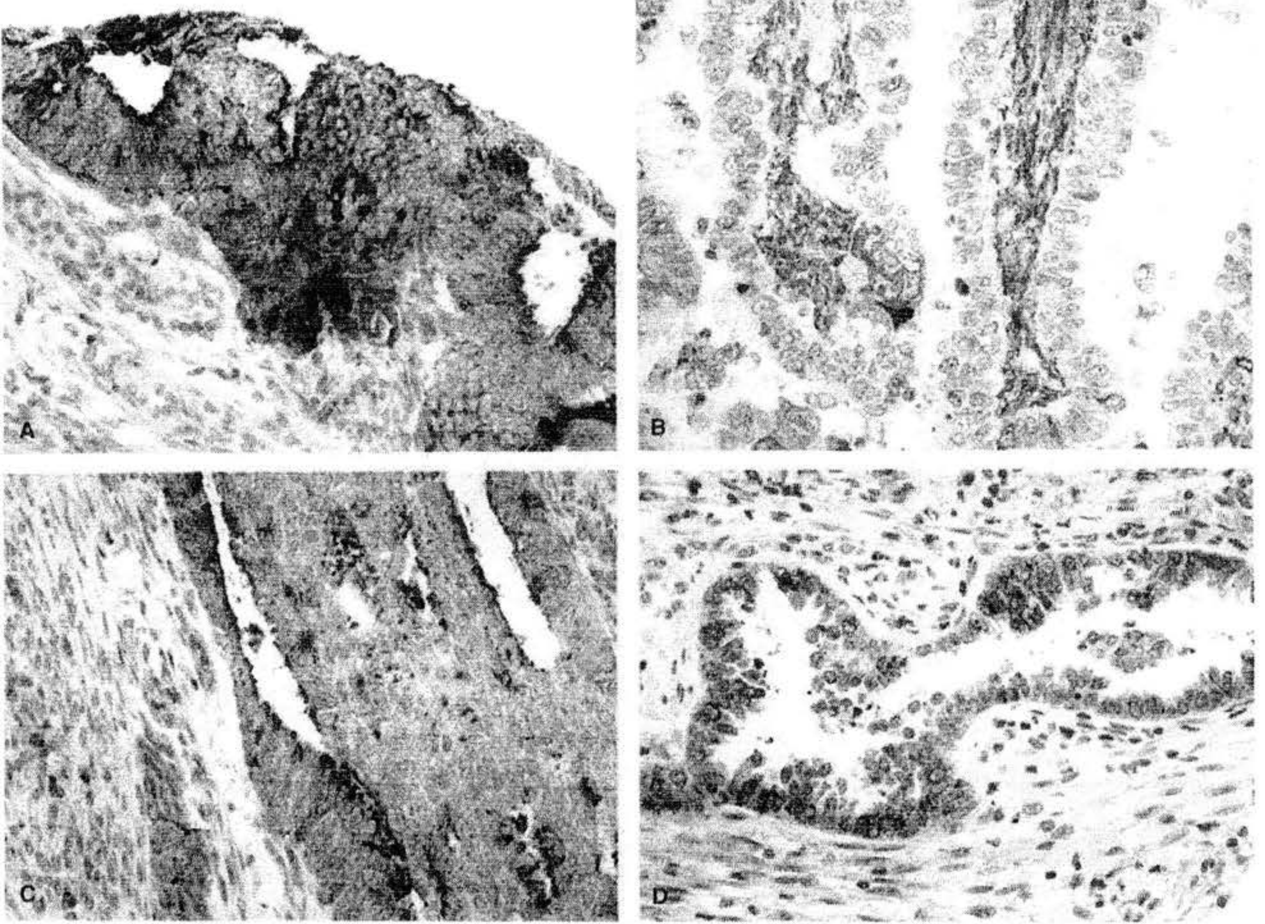

Figure 1. Expression of matrix metalloproteinases (MMPs). A, MMP-2 in endometrioid endometrial carcinoma (EEC). B, MMP-2 in uterine serous carcinoma (USC). C, MMP-7 in EEC. D, MMP-7 in USC.

There was significantly greater expression of β-catenin in carcinoma cells at the invasive edge compared with those in the viable central part of the tumour ($P = 0.03$, Wilcoxon signed-ranks test, Table 2). However, all β-catenin expression was membranous (Figure 2D).

There was significantly greater expression of MMP-2 in stromal cells compared with carcinoma cells in both the viable central part of the tumour and at the invasive edge ($P < 0.05$ and $P = 0.001$, respectively, Wilcoxon signed-ranks test, Table 3). In contrast, there was significantly less expression of MMP-7 in the stromal cells compared with the carcinoma cells in both the viable central part of the tumour and at the invasive edge ($P < 0.05$ and $P = 0.001$, respectively, Wilcoxon signed-ranks test, Table 3). There was significantly less MMP-9 expression in carcinoma cells compared with stromal cells in the viable central part of the tumour ($P = 0.02$, Wilcoxon signed-ranks test, Table 3), but there was no significant difference in MMP-9 expression between these cells at the invasive edge.

Endometrioid endometrial carcinoma

No significant differences in expression of any of the proteins (MMPs-2, -7, -9 or β-catenin) were noted in either carcinoma cells or stromal cells at the invasive edge compared with those in the viable central part of the tumour (Table 2). Expression of all the MMPs was both cytoplasmic and membranous (Figures 1A,C and 2A, respectively). Two of the 20 EEC cases showed nuclear β-catenin expression (Figure 2C). The remainder showed membranous staining.

There was no significant difference in expression of MMP-2 between carcinoma and stromal cells in either the viable central part of the tumour or at the invasive

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COLOUR FIG.

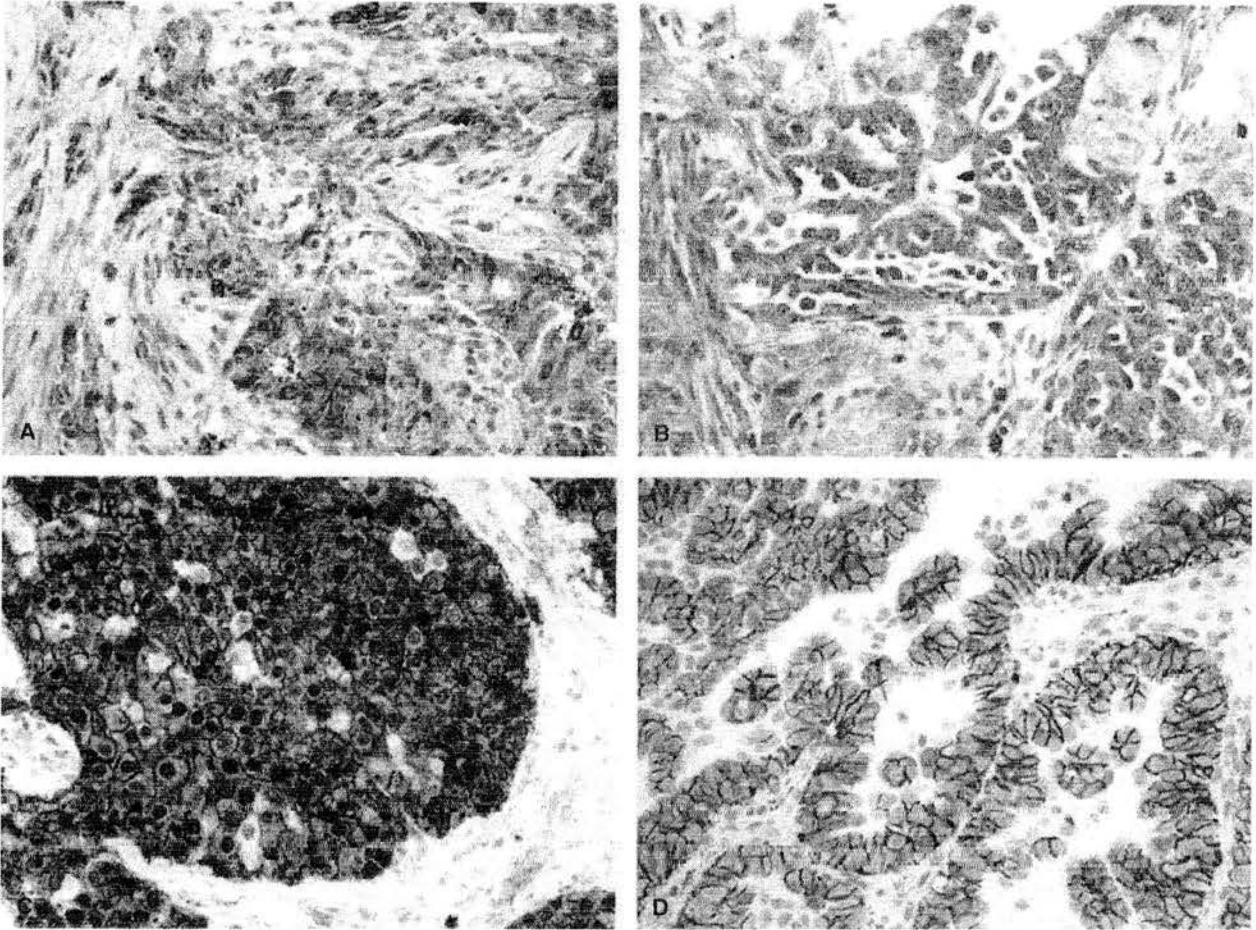

Figure 2. Expression of matrix metalloproteinases (MMPs). A, MMP-9 in endometrioid endometrial carcinoma (EEC). B, MMP-9 in uterine serous carcinoma (USC). C, Nuclear β-catenin in EEC. D, Membranous β-catenin in USC.

Table 3. Variation in matrix metalloproteinase (MMP) expression between tumour and stromal cells in uterine serous carcinoma

	Main tumour		Invasive edge	
Antibody	Tumour cells	Stromal cells	Tumour cells	Stromal cells
MMP-2	0.53*	1.68*	0.79†	1.54†
MMP-7	1.81**	1.27**	1.69††	1.21††
MMP-9	0.90***	1.24***	1.08	1.22

*$P < 0.05$ (Wilcoxon signed-ranks test); **$P < 0.05$ (Wilcoxon signed-ranks test); ***$P = 0.02$ (Wilcoxon signed-ranks test).
†$P = 0.001$ (Wilcoxon signed-ranks test); ††$P = 0.001$ (Wilcoxon signed-ranks test).

Table 4. Variation in matrix metalloproteinase (MMP) expression between tumour and stromal cells in endometrioid endometrial carcinoma

	Main tumour		Invasive edge	
Antibody	Tumour cells	Stromal cells	Tumour cells	Stromal cells
MMP-2	1.43	1.42	1.69	1.46
MMP-7	1.77*	1.52*	1.61	1.51
MMP-9	1.14**	2.51**	1.09†	1.76†

*$P = 0.018$ (Wilcoxon signed-ranks test); **$P = 0.011$ (Wilcoxon signed-ranks test).
†$P = 0.012$ (Wilcoxon signed-ranks test).

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edge (Table 4). There was significantly greater expression of MMP-7 in carcinoma cells compared with stromal cells in the viable central part of the tumour ($P = 0.018$, Wilcoxon signed-ranks test, Table 4), but there was no significant difference in expression between the cell types at the invasive edge. Significantly greater expression of MMP-9 was noted in stromal cells compared with carcinoma cells in both the viable central part of the tumour and at the invasive edge ($P = 0.011$ and $P = 0.012$, respectively, Wilcoxon signed-ranks test, Table 4).

Discussion

USC is an aggressive endometrial tumour with a notable propensity for lymphovascular space invasion and a poor prognosis. We performed this study to determine whether MMPs have a role in their increased invasiveness in comparison with EEC. We confirmed the findings of Misugi *et al.*[38] and Moreno-Bueno *et al.*[50] and demonstrated MMPs-2, -7 and -9 and β-catenin to be present in both USC and EEC.

In addition, the present study looked for differences in expression of these proteins between the different histological tumour types and within the tumours (the viable central part of the tumour and at the invasive edge). We demonstrated MMP-2 expression in both EEC and USC carcinoma cells, confirming the findings of Park *et al.*[48] Park *et al.*[48] demonstrated that synthesis and secretion of stromal MMP-2 is up-regulated by β-oestradiol. In addition, they showed that the invasiveness of cells from an endometrial adenocarcinoma cell line was enhanced by recruitment of MMP-2 secreted by endometrial stromal cells to their cell membrane, which was further enhanced by the presence of β-oestradiol.

The finding of significantly higher MMP-2 expression in EEC carcinoma cells compared with those in USC may partly reflect the different biological properties of these tumours. EEC is a tumour which usually arises on a background of oestrogen excess, which commonly expresses oestrogen receptor and can produce endogenous oestrogen using aromatase.[51] On the other hand, USC usually arises from atrophic endometrium, is generally oestrogen receptor-negative and does not produce endogenous oestrogen. Park *et al.*[48] have demonstrated that β-oestradiol stimulates MMP-2 secretion in adenocarcinoma cell lines; it is therefore possible that the differential secretion may be due to increased endogenous oestrogen in EEC compared with USC. We found significantly higher oestrogen receptor positivity in our EEC cases compared with USC (results not shown).

We found similar levels of MMP-2 in EEC carcinoma and stromal cells and although there was increased expression of MMP-2 in carcinoma cells at the invasive edge of the tumour, this was not statistically significant. However, we did find a significantly higher level of MMP-2 in USC stromal cells compared with carcinoma cells both in the viable central part of the tumour and at the invasive edge. In addition, the USC carcinoma cells showed a significant increase in MMP-2 expression at the invasive edge compared with the viable central part of the tumour. MMP-2 is synthesized and secreted by stromal cells and then binds to the TIMP-2:MT-MMP complex on the carcinoma cell surface;[32] this difference in expression raises the possibility that an invasive subclone of USC uses increased carcinoma cell binding of MMP-2 to equip the cells with the proteases required for invasion.

We did not demonstrate any intertumoral or intratumoral differences in expression of MMP-7. There was significantly more expression of MMP-7 in carcinoma cells compared with stromal cells in both USC and EEC. Our findings are in keeping with the published literature that MMP-7 is generally expressed in tumours.[38–41] As this study examined high-grade endometrial cancers only, we could not confirm or refute the findings of Misugi *et al.*,[38] who showed differential expression of MMP-7 between the different grades of EEC.

There was no significant difference in β-catenin expression between EEC and USC. However, we did find that β-catenin expression was increased significantly at the invasive edge of USC. All USC cases demonstrated membranous expression of β-catenin and only two EEC cases (10%) demonstrated nuclear expression. All other cases showed membranous expression. Our finding that 10% of EEC cases showed nuclear expression is less than that reported by Moreno-Bueno *et al.*,[52] who found that nuclear β-catenin expression was present in 31% of EECs and 3% of non-endometrioid endometrial carcinomas.[53] It is possible that this discrepancy is due to our case selection of only high-grade EEC, and *β-catenin* mutations may be more important in the development of Grade 1 and Grade 2 EEC.

As well as roles associated with cadherin function, β-catenin is also involved in intracellular signalling, as it is a member of the Wnt pathway. The activation of the Wnt signalling pathway due to *β-catenin* mutations has been implicated in the development of some endometrial carcinomas. However, up to 25% of endometrial carcinomas have β-catenin nuclear accumulation without evidence of *β-catenin* mutations,

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suggesting alterations in other molecules that can modulate the Wnt pathway such as APC, γ-catenin, AXIN1 and AXIN2. In ovarian cancer the presence of nuclear β-catenin is an indicator of good prognosis, but whether this has the same effect in endometrial cancers is as yet unknown.

Crawford *et al.*[54] have reported a positive correlation between nuclear β-catenin protein levels and MMP-7 transcripts in colonic carcinoma. They did not find any MMP-7 expression in cells that lacked β-catenin protein accumulation. Mylona *et al.*[55] have demonstrated an inverse correlation with nuclear β-catenin expression in breast carcinoma. We did not demonstrate any inverse or direct correlation between β-catenin expression and any of the MMPs. This may be due to the relatively small numbers of cases involved. Alternatively, this may be because the studies that have demonstrated either inverse or direct correlations have focused on colonic carcinoma where β-catenin expression is nuclear, reflecting mutation in the *β-catenin* gene, and the studies by Mylona *et al.*[55] in breast carcinoma also focused on *β-catenin* mutation.

We found significantly higher expression of MMP-9 in EEC compared with USC and also significantly higher expression of MMP-9 in stromal cells compared with carcinoma cells in the viable central part of the tumour in both EEC and USC. In addition, there was significantly higher expression of MMP-9 in stromal cells compared with carcinoma cells at the invasive edge of the EEC cases. However, there was no significant difference in MMP-9 expression between the invasive edge stromal cells and carcinoma cells in USC. MMP-9 is normally synthesized and secreted by stromal cells and binds to CD44 on carcinoma cells.[32] The loss of this differential expression between carcinoma cells and stromal cells at the invasive edge of USC cases may indicate that MMP-9 synthesis and secretion by stromal cells is unchanged at the invasive edge of the tumour, but binding of MMP-9 by the invading carcinoma cells is increased, perhaps enhancing their invasive potential.

Hui *et al.*[16] have recently demonstrated differences in outcome between patients with Stage 1a USC arising in a polyp and in the 'normal' endometrium. In view of our results showing differences in expression of MMPs-2 and -9 between the main part of the tumour and the invasive edge, it would be interesting to examine more closely cases of endometrial intraepithelial carcinoma and Stage 1a USC.

In summary, this immunohistochemical study has confirmed that MMPs-2, -7 and -9 and β-catenin are present in endometrial carcinomas and specifically in both EEC and USC. We found significantly higher expression of MMPs-2 and -9 in EEC compared with USC. This may be due to the known relationship between these MMPs and oestrogen status. Despite the lower expression of MMP-2 and MMP-9 in USC compared with EEC, we did show increased expression of MMP-2 and MMP-9 by carcinoma cells at the invasive edge of USC. This is probably due to increased binding to carcinoma cells of MMPs secreted by the stromal cells. This difference in staining pattern between the viable central part of the tumour and the invasive edge supports the theory of an invasive subclone of USC acquiring activated proteases in order to invade.

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Appendix 2

Individual results of P53 from whole sections (WS), tissue microarray (TMA) and compressed scores (POS NEG) for Chapter 3 TMA Validation.

Number	WS	WS POS NEG	TMA POS NEG	SCORE 1	SCORE 2	SCORE 3	SCORE 4	MISSING
1	.	.	2	0	0	3	4	1
2	2	2	2	0	1	0	1	2
3	.	.	2	0	0	0	4	0
4	1	2	2	0	6	0	0	0
5	3	2	2	0	0	0	2	0
6	2	2	1	4	0	0	0	0
7	.	.	.	0	0	0	0	4
8	.	.	2	0	2	1	0	1
9	.	.	2	4	0	2	0	0
10	.	.	2	0	0	0	6	0
11	3	2	2	0	0	2	0	0
12	2	2	2	3	2	0	0	1
13	2	2	2	0	1	1	0	0
14	3	2	2	0	0	0	3	1
15	3	2	2	0	0	0	5	1
16	3	2	2	0	0	0	2	0
17	3	2	2	0	0	0	3	1
18	3	2	2	0	0	0	8	1
19	2	2	2	0	4	0	0	0
20	.	.	2	0	0	0	4	0
21	.	.	2	0	0	0	4	0
22	.	.	.	.	.	.	.	.
23	.	.	2	0	0	0	6	0
24	.	.	2	0	0	0	4	0
25	.	.	2	1	1	0	0	2
26	.	.	1	4	0	0	0	0
27	3	2	2	0	0	8	0	0
28	0	1	1	2	0	0	0	2
29	2	2	1	4	0	0	0	0
30	.	.	2	0	0	2	0	0
31	3	2	2	0	0	0	4	2
32	2	2	2	0	0	2	0	0
33	3	2	2	0	0	3	2	1
34	3	2	2	0	0	0	3	1
35	.	.	2	0	1	2	0	1
36	.	.	.	0	0	0	0	2
37	2	2	2	0	1	3	0	2
38	.	.	2	0	2	0	0	2
39	.	.	2	0	0	0	10	0
40	.	.	2	0	0	0	3	1
41	3	2	2	0	0	0	4	0
42	3	2	2	0	0	0	4	0
43	0	1	1	3	0	0	0	1
44	3	2	2	0	0	0	4	0

Number	WS	WS POS NEG	TMA POS NEG	SCORE 1	SCORE 2	SCORE 3	SCORE 4	MISSING
45	2	2	2	0	2	0	0	2
46	3	2	2	0	0	0	4	0
47	3	2	2	0	0	0	2	2
48	3	2	2	0	0	0	7	1
49	.	.	1	2	0	0	0	0
50	2	2	2	0	0	2	0	0
51	2	2	1	2	0	0	0	0
52	3	2	2	0	0	0	6	0
53	3	2	2	0	0	0	1	1
54	2	2	.	.	.	.	.	.
55	.	.	.	.	.	.	.	.
56	2	2	.	0	0	0	0	2
57	3	2	1	2	0	0	0	0
58	.	.	2	0	1	1	0	0
59	3	2	2	0	0	0	2	0
60	3	2	2	0	0	0	4	0
61	3	2	2	0	0	1	0	1
62	2	2	1	2	0	0	0	2
63	2	2	2	1	1	0	0	2
64	.	.	2	0	6	0	0	0
65	.	.	2	1	4	1	0	0
66	.	.	2	0	0	1	2	1
67	.	.	2	0	0	0	6	0
68	.	.	2	0	0	0	3	1
69	.	.	2	0	0	0	4	0
70	.	.	2	0	0	0	3	1
71	.	.	1	2	0	0	0	0
72	.	.	2	0	0	0	4	0
73	.	.	2	0	0	1	6	1
74	.	.	2	0	0	0	2	2
75	.	.	2	0	3	1	0	0
76	.	.	1	5	0	0	0	1
77	.	.	2	0	0	2	2	0
78	.	.	2	0	5	1	0	0
79			1	2	0	0	0	2
80	3	2	.	0	0	0	0	2

Appendix 3

Individual results of Oestrogen receptor status (ER) from whole sections (WS), tissue microarray (TMA) and compressed scores (POS NEG) for Chapter 3 TMA Validation.

Case number	WS total	TMA total	WS POS NEG	TMA POS NEG
1	.	2.63	.	1
2	0		1	
3	.	4.5	.	2
4	4	2.17	1	1
5	6	6	2	2
6	8	5.25	2	2
7	.	.	.	.
8	.	6	.	2
9	.	6.66	.	2
10	.	3.5	.	1
11	5	6	2	2
12	[illegible]	2.8	2	1
13	4	0	1	1
14	0	0.5	1	1
15	7	[illegible]	2	2
16	4	0	1	1
17	4	3.33	1	1
18	0	1	1	1
19	4	2	1	1
20	.	4	.	1
21	.	3	.	1
22	.	.	.	.
23	.	4.23	.	2
24	.	8	.	2
25	.	8	.	2
26	.	5.75	.	2
27	0	0	1	1
28	0	.	1	.
29	0	0	1	1
30	.	6	.	2
31	8	7.75	2	2
32	5	5.5	2	2
33	6	6.8	2	2
34	3	3	1	1
35	.	7	.	2
36	.	.	.	.
37	4	1.25	1	1
38	.	4.5	.	2
39	.	6.6	.	2
40	.	5.33	.	2
41	8	7.25	2	2
42	6	0.5	2	1
43	3	3.33	1	1
44	3	1	1	1

Case number	WS total	TMA total	WS POS NEG	TMA POS NEG
45	8	8	2	2
46	5	6.75	2	2
47	4	2.5	1	1
48	7	6.14	2	2
49	.	7	.	2
50	6	5.5	2	2
51	5	2.5	2	1
52	6	2.37	2	1
53	6	0	2	1
54	7	.	2	.
55	.	.	.	.
56	4	.	1	.
57	0	0	1	1
58	.	3	.	1
59	7		2	
60	6	7	2	2
61	3	.	1	.
62	7	5	2	2
63	6	6.33	2	2
64	.	7.83	.	2
65	4	3.2	1	1
66	.	.	.	.
67	.	7	.	2
68	.	5	.	2
69	.	0	.	1
70	.	5	.	2
71	.	3	.	1
72	.	5.33	.	2
73	.	3.13	.	1
74	.	5	.	2
75		7.25		2
76	.	6.25	.	2
77	.	6	.	2
78	.	7.8	.	2
79	.	7	.	2
80	5	.	2	.